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GESTATIONAL VARIATIONS IN THE COMPOSITION OF THE FOETAL FLUIDS AND FOETAL URINE IN THE SHEEP

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was from 45 to 142 days (the gestation period in this breed being about 145 days). Anaesthesia was induced by spinal administration of procaine. The animal was placed in the supine position in a bath of saline at 38° C, the head and thorax being elevated to an angle of 45°, and the foetus was delivered into the bath by Caesarean section (Huggett, 1927). Intravenous supplements of sodium thiopentone (Pentothal, Abbott Laboratories) were given to the ewe if and when required in order to maintain a light plane of general anaesthesia.

Maternal blood was obtained from the dorsalis pedis artery, foetal blood samples from one of the four umbilical vessels. The blood samples were withdrawn into centrifuge tubes containing dry heparin and the plasma obtained by centrifuging for 30 min at 3000 r.p.m. Amniotic and allantoic fluids and maternal urine samples were removed by puncturing the respective sacs and bladder and withdrawing the samples by syringe. Foetal urine samples were obtained from the foetal bladder after the abdomen had been opened under local anaesthesia.

Determinations of the osmotic pressure were carried out with a cryoscope having a Stantel Thermistor as the temperature-sensitive element.

Total nitrogen and non-protein nitrogen (after protein precipitation by trichloroacetic acid) were determined by the Markham micro-Kjeldahl method; amino acid nitrogen by the method of Pope & Stevens (1939) after heat coagulation of the protein; urea by the micro-diffusion

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GESTATIONAL VARIATIONS IN THE COMPOSITION OF THE FOETAL FLUIDS AND FOETAL URINE IN THE SHEEP

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Early investigations into the composition of the foetal fluids have been reviewed by Needham (1931). More recently other workers, notably Malan, Malan & Curson (1937), Cloete (1939) and McDougall (1949) have examined the composition of the fluids in the sheep, but the relationship of these to the plasmas on the one hand and the urines on the other have been largely neglected in the past. The purpose of the present investigation was to re-examine the composition of the foetal fluids and to make comparisons of the concentration of the constituents present with those in the maternal and foetal plasmas and the maternal and foetal urines.

METHODS

The animals were Welsh Mountain ewes of known conceptual age. The range of foetal age examined was from 45 to 142 days (the gestation period in this breed being about 145 days). Anaesthesia was induced by spinal administration of procaine. The animal was placed in the supine position in a bath of saline at 38° C, the head and thorax being elevated to an angle of 45°, and the foetus was delivered into the bath by Caesarean section (Huggett, 1927). Intravenous supplements of sodium thiopentone (Pentothal, Abbott Laboratories) were given to the ewe if and when required in order to maintain a light plane of general anaesthesia.

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Determinations of the osmotic pressure were carried out with a cryoscope having a Stantel Thermistor as the temperature-sensitive element.

Total nitrogen and non-protein nitrogen (after protein precipitation by trichloroacetic acid) were determined by the Markham micro-Kjeldahl method; amino acid nitrogen by the method of Pope & Stevens (1939) after heat coagulation of the protein; urea by the micro-diffusion method (Conway, 1947); sulphate by the barium sulphate turbidimetric method of Sperber (1948); total and inorganic phosphate, creatinine and uric acid by the methods of King (1946). The Na^+ and K^+ concentrations were determined by flame photometer, Cl^- by the method of Sanderson (1952); total reducing substance was estimated by the method of Somogyi (1952) and fructose as described by Bacon & Bell (1948), after deproteinization with NaOH and $ZnSO_4$. Electrometric determination of pH (using a Cambridge pH meter) was carried out on samples which were collected under liquid paraffin.

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The accuracy of the estimations of osmolarity of sodium, potassium, chloride, urea and fructose concentrations was of the order of 3%, and the remaining estimations (some of which relied on difference methods) were of the order of 5–10%.

RESULTS

The results of osmotic pressure determinations are shown in Table 1. The osmotic pressure of the foetal plasma is very similar to that of the maternal plasma.

The foetal bladder urine is considerably hypotonic with respect to the plasma and is typically in the range 50–80% of the tonicity of foetal plasma, while the osmotic pressure of the maternal urine varied up to five times that of the plasma.

TABLE 1. Average osmotic pressure values (m-osmole/l.) in the plasmas, foetal fluids and foetal urine

Foetal age (days)	Maternal plasma	Foetal plasma	Amniotic fluid	Allantoic fluid	Foetal urine
45– 69	335 (6)	335 (4)	333 (6)	248 (6)	—
81– 93	332 (4)	344 (5)	327 (5)	286 (5)	239 (4)
104–117	339 (5)	353 (5)	307 (5)	288 (5)	207 (2)
130–142	339 (4)	353 (4)	303 (4)	322 (4)	166 (1)

Figures in parentheses indicate number of observations.

The relationship of the cryoscopic values in the two foetal fluids with foetal age are seen in Fig. 1. The allantoic fluid is initially hypotonic but the tonicity rises towards term, whereas the amniotic fluid is approximately isotonic in the early stages of gestation with hypotonic values being found after about 90 days. The foetal bladder urine was found to be hypotonic with respect to the foetal fluids (except in one case).

The total nitrogen concentration in the foetal plasma and in the amniotic fluid shows a mutual correlation as well as a positive correlation with foetal age (Fig. 2). That in the maternal plasma varied from 880 to 1370 mg/100 ml., mean 1117 mg/100 ml.

The total nitrogen of the allantoic fluid remains considerably higher than that of the amniotic over the whole age range examined and varies from 73 to 468 mg/100 ml., with an average value of 273 mg/100 ml. The values show no definite relationship to foetal age except perhaps in foetuses younger than 57 days. A positive correlation of total nitrogen in the foetal urine with foetal age (81–142 days) was found (correlation coefficient, r, $=0{\cdot}875$, $P<0{\cdot}001$), the total nitrogen increasing from 55 to 193 mg/100 ml.

The non-protein nitrogen (N.P.N.) of the foetal plasma was found to be in all cases greater (mean value 45, range 37–50 mg/100 ml.) than that present in the maternal plasma. The amniotic N.P.N. was usually less, while the allantoic N.P.N. was considerably higher, than that of the foetal plasma. Amino acid nitrogen concentration was consistently highest in the allantoic fluid. One sample of foetal plasma estimated for amino acid nitrogen gave a value of 26·6 mg/100 ml. compared with a mean maternal plasma concentration of 14·8 mg/100 ml. (Table 2).

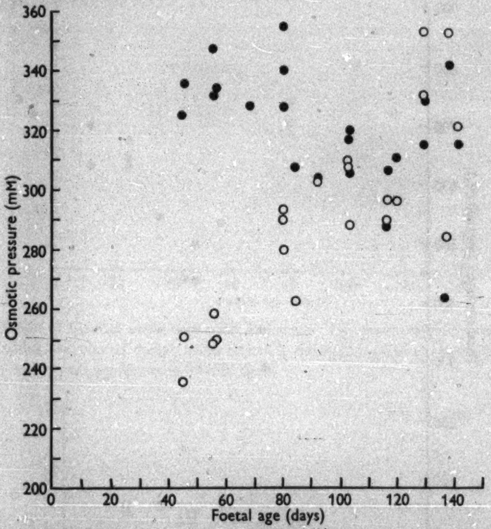

Fig. 1. Correlation of osmotic pressure in the foetal fluids with foetal age. ●, Amniotic fluid; ○, allantoic fluid. From 45 to 142 days the osmotic pressure in the allantoic fluid rises and gives a significant positive correlation with foetal age ($r = 0·846$, $P < 0·001$). The osmotic pressure in the amniotic fluid shows a significant negative correlation ($r = 0·563$, $P < 0·01$) over the same age range.

The urea concentration in bladder urine increases with foetal age (Fig. 3). This rise in urea concentration is reflected in the urea concentration of the foetal fluids (Fig. 4).

While the allantoic urea concentration is variable (mean 64, range 12–140 mg/100 ml.), that of the amniotic fluid shows a progressive increase in concentration with gestation age. The permeability of the placenta to urea is reflected in the correlation of the concentrations of maternal plasma urea with foetal plasma urea (Fig. 5).

The creatinine values are shown in Table 3. It will be seen that the creatinine concentration in the amniotic fluid exceeds that found in the foetal plasma in the older foetuses but is less than that of the foetal urine. Creatinine values of the allantoic fluid are higher than those of the urine and show a positive correlation with foetal age.

The uric acid concentration of the allantoic fluid in five animals examined exceeded that of the amniotic fluid, the ranges being 1·4–57·4 mg/100 ml. and 0·8–5·5 mg/100 ml. respectively.

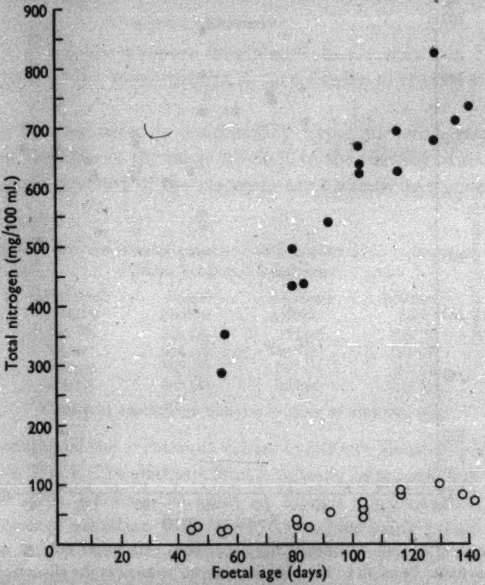

Fig. 2. Correlation of the total nitrogen in the foetal plasma and amniotic fluid with foetal age. ●, Foetal plasma; ○, amniotic fluid. Over the age range examined a positive correlation of the total nitrogen in the foetal plasma to foetal age is present ($r = 0{\cdot}942$, $P < 0{\cdot}001$). A similar correlation is present in the amniotic fluid ($r = 0{\cdot}892$, $P < 0{\cdot}001$).

Table 2. Non-protein and amino acid nitrogen in the maternal plasma and foetal fluids (mg/100 ml.)*

	Maternal plasma	Amniotic fluid	Allantoic fluid
Non-protein nitrogen	30 (17–37) *17*	26 (20–40) *7*	210 (136–268) *5*
Amino acid nitrogen	14·8 (7·3–20·0) *5*	3·9 (3·0–4·4) *5*	53 (21·8–75·6) *4*

* Mean (range); no. of observations in italics.

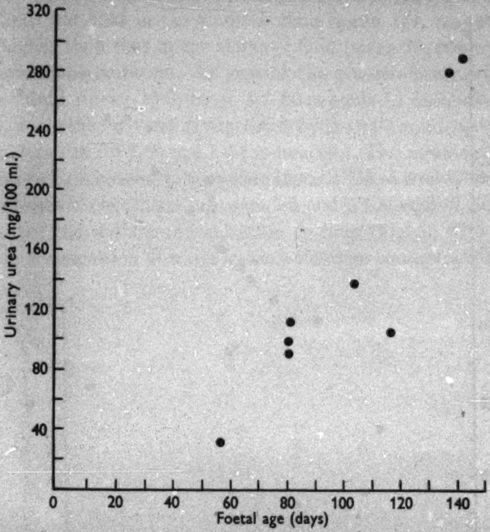

Fig. 3. Correlation of bladder urine urea with foetal age. The concentration of urea present in initial bladder samples of foetal urine shows a significant positive correlation ($r = 0.910$, $P < 0.01$) with foetal age between 57–142 days.

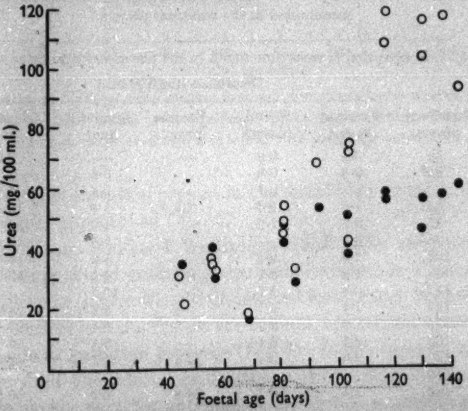

Fig. 4. Correlation of urea in the foetal plasma and amniotic fluid with foetal age. ●, Foetal plasma; ○, amniotic fluid. A significant positive correlation of urea in foetal plasma ($r = 0.718$, $P < 0.001$) and amniotic fluid ($r = 0.893$, $P < 0.001$) exists over the age range 45–142 days.

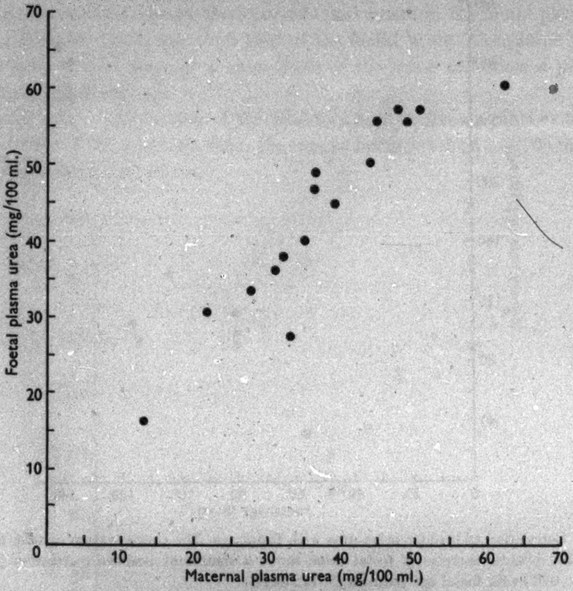

Fig. 5. Relationship of the foetal plasma urea with the concentration encountered in the maternal plasma.

Table 3. Concentration of creatinine found in the plasmas, foetal urine and fluids.

Foetal age (days)	Creatinine (mg/100 ml.) Maternal plasma	Foetal plasma	Foetal urine	Amniotic fluid	Allantoic fluid
69	0·4	0·8	—	—	2·0
81*	0·4	0·8	—	0·4	3·7
		0·1	—	0·8	4·0
81	1·2	2·7	2·8	1·8	13·0
85	0·8	1·2	—	1·9	14·3
93	0	—	—	4·3	13·2
104	2·8	2·6	—	4·9	14·0
104	1·3	2·9	—	2·9	10·7
117	0·9	1·5	—	10·2	—
117	1·4	2·7	6·4	6·8	24·9
130	1·2	2·7	—	6·4	27·5
130	2·3	3·1	—	9·4	59·0
137	2·0	3·4	24·4	9·1	44·0
140	—	—	—	—	—
142	2·5	4·2	28·8	6·1	66·0

* Twins.

No consistent trend is discernible in the sodium concentrations in either of the foetal fluids but that in the amniotic fluid (mean 114, range 85–146 m-equiv/l.) is higher than that in the allantoic fluid (mean 70, range 32–131 m-equiv/l.) throughout gestation. In general the potassium concentration of the amniotic fluid (mean 10·6, range 4·7–30 m-equiv/l.) declines as term is approached, while the converse is suggested by the values encountered in the allantoic fluid (mean 10·2, range 1–33 m-equiv/l.). The mean maternal and foetal plasma sodium concentrations were 160 and 152 m-equiv/l. respectively, while the potassium concentrations were 6·0 and 6·7 m-equiv/l. respectively. In foetal urine, the sodium concentration declines (Fig. 6) with foetal age while there is a suggestion of a rise in the potassium concentration.

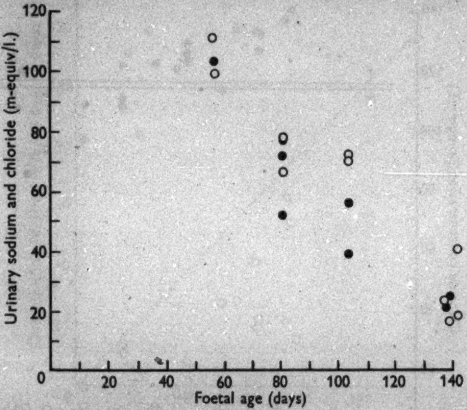

Fig. 6. Correlation of bladder urine sodium and chloride concentrations with foetal age. ○, Sodium; ●, chloride. There is a significant negative correlation of sodium ($r=0.938$, $P < 0.001$) and of chloride ($r=0.944$, $P < 0.001$) with foetal age between 57 and 142 days.

The chloride concentration present in the foetal bladder urine shows a steep decline as term is approached (Fig. 6).

Alexander, Nixon, Widdas & Wohlzogen (1955, 1958) have shown that the sheep foetus is capable of producing considerable amounts of urine. The introduction of this urine, low in Na and Cl, into the foetal fluids theoretically would result in a progressive reduction in the sodium and chloride concentration of the foetal fluids as gestation proceeds (Fig. 7). It will be seen, however, that the amniotic fluid chloride concentration, initially higher than that in the plasma, falls slowly as term approaches, being below plasma chloride concentration after 100 days, whilst the allantoic chloride concentration falls sharply until it reaches about 6 m-equiv/l. in the region of 90 days and then remains relatively unchanged until term. The sodium concentrations of the foetal fluids do not reflect the changes in the chloride concentrations.

If the amniotic and allantoic fluid chloride concentrations are plotted against urinary chloride concentration, as in Fig. 8, then the values are found to lie on opposite sides of the diagonal line which would represent the expected concentration if either the amniotic or allantoic fluid was merely unchanged urine collected in the respective sacs. The points for amniotic fluid lie above this line and this is consistent with the view that in amniotic fluid urine only represents part of the volume and that there is some other contribution to the formation of this fluid. The fact that the points for allantoic fluid all lie below the line indicates either an addition of water or a removal of chloride. Since the allantoic fluid is hypotonic throughout gestation and osmotic forces would favour a removal of water, the addition of water seems unlikely. The absorption of Cl ions from the allantoic fluid would appear the more likely hypothesis but, as both plasma and amniotic fluids have a higher chloride concentration, any movement of chloride towards those fluids would be against a concentration gradient, and it would be necessary to postulate an active process or a Donnan-type equilibrium.

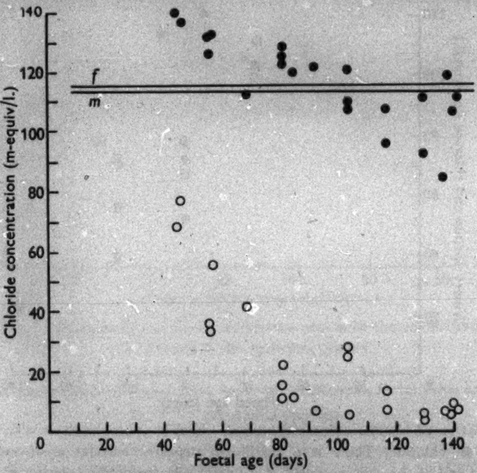

Fig. 7. Decline of chloride concentrations in the foetal fluids with increasing foetal age. ●, Amniotic fluid; ○, allantoic fluid. Chloride concentrations in both fluids show a significant negative correlation with foetal age between 45 and 142 days; amniotic fluid ($r=0{\cdot}798$, $P<0{\cdot}001$), allantoic fluid ($r=0{\cdot}809$, $P<0{\cdot}001$). *f*, mean foetal plasma chloride; *m*, mean maternal plasma chloride.

The foetal plasma phosphate concentrations are slightly above those found in the simultaneous maternal plasma sample. Amniotic fluid phosphorus values are in the main less than those found in the allantoic fluid. When attempting phosphorus determinations in the allantoic fluid, turbidity was encountered, similar to that observed by McDougall (1949).

The sulphate concentration present in the allantoic fluid was found to be considerably higher than that of the amniotic fluid.

The phosphate and sulphate concentrations together with pH values are shown in Table 4.

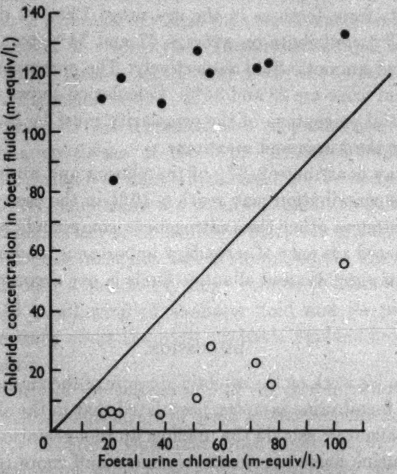

Fig. 8. Relationship of the urinary chloride with that encountered in the foetal fluids. ●, Amniotic fluid; ○, allantoic fluid. The diagonal line illustrates the relationship that would be obtained if the foetal fluids were composed of unaltered foetal urine.

Table 4. Total and inorganic phosphorus, sulphate and pH values found in the plasmas, urine and foetal fluids (mg/100 ml., except pH)*

	Maternal plasma	Foetal plasma	Foetal urine	Amniotic fluid	Allantoic fluid
Total P	11·0 (7·9–13·4) *13*	11·7 (9·6–13·9) *14*	4·8 (1·1–11·2) *5*	2·7 (0·6–8·8) *17*	7·3 (1·9–23·8) *16*
Inorganic P	7·8 (4·4–11·3) *8*	7·4 (5·6–8·5) *4*	—	2·3 (1·5–4·1) *9*	—
Inorganic SO_4	—	—	—	3·6 (0·5–8·7) *9*	55·1 (22·9–88·0) *9*
pH	7·36 (7·2–7·57) *4*	7·55 *1*	—	7·37 (6·95–7·52) *6*	6·92 (6·1–7·34) *5*

* Mean (range); no of observations in italics.

The wide range of concentrations of glucose and fructose in the foetal blood and in the foetal fluids was of the same order as that encountered by Barklay, Haas, Huggett, King & Rowley (1949).

An attempt to assess the relative proportions of electrolytes and non-electrolytes in the fluids has been made by considering the contribution of the osmotically more important substances to the total osmolarity. The great variability of composition, particularly in urine samples, makes this assessment subject to considerable error, but the data illustrate the different patterns of plasma and amniotic fluid on the one hand and allantoic fluid and urine on the other.

Thus in results from foetuses in the age range 130–142 days, electrolytes (Na^+, K^+ and Cl^-) contribute on average 77 and 74 % to the osmolarity of foetal plasma and amniotic fluid respectively. The corresponding figures for allantoic fluid and urine are 28 and 34 %. Calculation shows that in the latter fluids a substantial percentage of the osmolarity must be due to nitrogenous substances other than urea and creatinine.

The sugars may contribute 2–3 % of the plasma and amniotic fluid osmolarity, while this contribution may reach 6–10 % in the case of allantoic fluid and urine. Substances other than nitrogenous compounds and sugars which have been estimated are only of secondary importance, and about 10–20 % of the osmolarity of amniotic and allantoic fluids is not accounted for.

DISCUSSION

Jacqué (1902), in his work on the osmotic pressure of the amniotic and allantoic fluids and a few foetal urine samples, postulated that in the sheep foetus urine passed into the allantoic sac via the urachus up to a gestation age of 90 days. Thereafter the urine passed progressively more and more into the amniotic sac with increasing foetal age, owing to occlusion of the urachus and the patency of the urethra. A further postulate was that because of the longer urethra in the male, the resistance to the passage of urine would be greater in this sex than that in the female at a comparable foetal age, and hence the proportion of urine flowing to the allantoic sac would be greater than that flowing to the amniotic; this, however, is not supported by the present data. The rate of urine formation by the foetal sheep kidney from at least 81 days to term is more than sufficient to account for the volumes of allantoic fluid encountered (Alexander *et al.* 1955, 1958). This ability on the part of the kidney to elaborate a fluid is present at a very early age. Davies (1952) has shown that the mesonephros of the embryo sheep is functionally active in embryos of only 4 mm in length (18 days post-fertilization); this activity is reflected by the appearance of fluid within the allantoic sac having a relatively high fructose concentration, yet at this stage of development little or no amniotic fluid is present. From observations on the pig, McCance & Dickerson (1957) have suggested that secretory processes centred in the allantoic membrane play a part initially in the formation of the allantoic fluid.

The production of a hypotonic urine as a mode of formation of allantoic fluid has interesting physiological consequences. In the early stages of gestation it may help to maintain the osmotic pressure of the foetal plasma at such a value that fluid is not lost to the maternal circulation. The hypotonic fluid, if stored in a sac of sufficient impermeability, could help in producing the general distension of the uterine cavity. That its contents remain hypotonic and reflect the tonicity of foetal urine strongly suggests a relatively small rate of water transfer, or alternatively that the rate of water transfer is balanced by an active removal of electrolytes. The present studies of concentrations suggest that an active removal of Cl^- may occur but so far there is no direct evidence of such a transfer.

As gestation proceeds the increased rate of production of urine and its elimination into a cul-de-sac of the type envisaged would result in the accumulation of a volume of fluid which could not be accommodated in the uterus and would be many times the volume of allantoic fluid actually found. It is clear that either an increased rate of reabsorption of fluid must occur from the allantoic sac or urine must pass into the amniotic sac. The latter alternative does not in itself offer a sufficiently large volume to accommodate the urine produced but the swallowing of amniotic fluid and its passage along the gastro-intestinal tract offers the most probable explanation of an increased rate of reabsorption.

The present study of the relationship of the concentrations in the various fluids coupled with measurements of urine production (Alexander *et al.* 1958) strongly supports Jacqué's view that from about 90 days' gestation age substantial quantities of urine must enter the amniotic sac via the urethra. In all our experiments it has been possible to demonstrate patency of the urethra but the control *in vivo* of sphincters or other factors affecting the relative distribution of fluid between amniotic and allantoic sacs is still unknown.

It is not asserted that foetal urine is the only source of amniotic fluid—all the evidence from the analysis of its composition is that it is more closely related to plasma than urine, particularly in younger foetuses. An admixture of a fluid of plasma-like composition with foetal urine could, however, best explain the findings for amniotic fluid for older foetuses.

Reynolds (1953) has shown that the secretions of the buccal cavity and respiratory tract can amount to 15 ml./hr in the foetal sheep. Thus, these and other foetal secretions may add appreciably to the volume and composition of the amniotic fluid. A further factor involved in the modification of the amniotic fluid composition is presumably brought about by the swallowing movements previously mentioned, which are observed in late foetuses. The stomachs of foetuses are frequently distended with fluid.

The reabsorption of fluid in the gastro-intestinal tract would make possible a maintenance of fluid balance in the rapidly growing foetus and its associated fluids, in which continued urine production was offset by a gastro-intestinal reabsorption, without postulating any significant change in the absorptive activities of the foetal membranes.

SUMMARY

1. The composition of foetal fluids in the sheep has been compared with that of maternal and foetal plasma and urine during the latter half of pregnancy.

2. Foetal urine is hypotonic, but otherwise its composition has typical urinary characteristics. This composition is closely related to that of allantoic fluid and is the probable source of that fluid.

3. Amniotic fluid has osmotic and other characteristics more closely related to those of foetal or maternal plasma but there is evidence of an admixture with foetal urine in the latter part of pregnancy which confirms the observations of Jacqué (1902).

4. Consideration of the volume of urine produced and evidence of its distribution to the amniotic sac suggest that ingestion and absorption of fluid by the gastro-intestinal tract may play a part in the regulation of the volume of foetal fluids in older foetuses.

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RENAL FUNCTION IN THE SHEEP FOETUS

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Leonardo da Vinci, at the commencement of the sixteenth century, recorded his observation that the human foetus produces urine *in utero* (quoted by Needham, 1931). More recently, analyses of foetal bladder samples in the sheep (Jacqué, 1902) and the human (Makepeace, Smith, Dailey & Carrol, 1931) have indicated that the foetus produces a hypotonic urine. The foetal rabbit, cat, opossum, chick and pig show glomerular excretion of ferrocyanide and tubular excretion of phenol red by both mesonephros and metanephros (Gersh, 1937), while human foetal concentration of uric acid has been shown by Kinnumen & Paalanen (1952). Wells (1946) and Daly, Wells & Evans (1947) have shown that within 24 hr of term the foetal rat's kidney is capable of responding to a subcutaneous injection of 0·15 ml. of 50% urea by diuresis. They also observed the blood: urine concentration ratios of urea and creatinine. Some quantitative observations on phenol sulphophthalein excretion by the foetal rabbit kidney during the last week of gestation have been made by Williamson & Hiatt (1947).

In none of the above experiments was the rate of urine production measured, and there would appear to be some difficulty in achieving such measurements in acute experiments on the smaller species. In experiments on sheep, however, it was found practicable to measure rates of foetal urine production in acute experiments during the latter half of pregnancy. This has enabled changes in the pattern of urine composition and flow to be investigated and urine analyses have been compared with similar tests carried out concurrently on blood and foetal fluids.

The relation of urine production to the changing composition in the foetal fluids is described in the preceding paper (Alexander, Nixon, Widdas & Wohlzogen, 1958). The present paper is concerned with the evidence which these experiments give regarding the development of function in the foetal sheep kidney between 61 and 142 days gestation age.

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Preliminary reports were given to the Physiological Society (Alexander, Nixon, Widdas & Wohlzogen, 1955) and the XXth International Physiological Congress (Alexander, Nixon, Widdas & Wohlzogen, 1956).

METHODS

The animals used were Welsh mountain ewes of known conceptual age. The age range investigated was from 61 to 142 days gestation age (full term in this breed being about 145 days). Procaine spinal anaesthesia was used, supplemented when necessary by intravenous thiopentone. The ewe was placed in a bath of saline at 38° C. The head and back were at an angle of 45° above the horizontal. The foetus was delivered by Caesarean section (Huggett, 1927), and, with the umbilical circulation maintained intact, retained close to the mother by means of a sling of lint strapped across her body. The foetal bladder was exposed under local anaesthesia by means of a mid-line incision below the umbilicus. The bladder was gently freed from the umbilical vessels running alongside it. After making a small incision in its wall the bladder was cannulated by means of a piece of glass tubing 2 in. long, with a constriction near one end, ligated in position. This glass tubing was attached to polythene tubing which lay along the body of the foetus, only the open end being maintained above the level of the saline. The urine was withdrawn at intervals from the tubing by means of a fine-drawn teat pipette which could pass to the level of the bladder. The volumes of all samples were recorded, together with the time of sampling.

In the initial experiments foetal blood samples were collected by repeated puncture of the umbilical veins. Later a fine polythene catheter was inserted via a tributary into the lumen of one of the two main umbilical veins, through which all the blood samples were obtained.

The methods used in all chemical analyses and determinations have been described in the preceding paper (p. 1).

RESULTS

Rate of urine flow

The average minute volume for each foetus was calculated from the volume of urine collected over timed collection periods within the first hour of the experiment. The flow (0·14 ml./min at 61 days) increased to a maximum (0·64 ml./min) at approximately 117 days, then fell again to 0·14 ml./min at 142 days (Table 1). When considered in relation to foetal weight, the minute

TABLE 1

Sheep no.	Sex	Foetal age (days)	Urine flow (ml./min)
926	F.	61	0·14 (0·1 –0·26)
942	F.	65	0·24 (0·08–0·37)
944	F.	71	0·18 (0·15–0·29)
807	F.	81	0·22 (0·08–0·35)
838	M.	85	0·24 (0·08–0·34)
821	F.	93	0·20 (0·05–0·6)
804	M.	104 A	0·43 (0·2 –0·67)
830	M.	104 B	0·54 (0·42–0·64)
820	M.	117 A	0·64 (0·4 –0·94)
814	M.	117 B	0·58 (0·2 –1·2)
934	F.	123	0·47 (0·17–0·57)
817	F.	130	0·46 (0·1 –0·77)
935	F.	137	0·12 (0·07–1·74)
911	M.	141	0·23 (0·16–0·51)
840	M.	142	0·14 (0·09–0·83)

Figures in parentheses indicate range of urine flow encountered during experiment. Except for sheep of 104 days and 117 days, which are subtitled A and B, all the sheep in this table will be referred to by their ages only in subsequent tables.

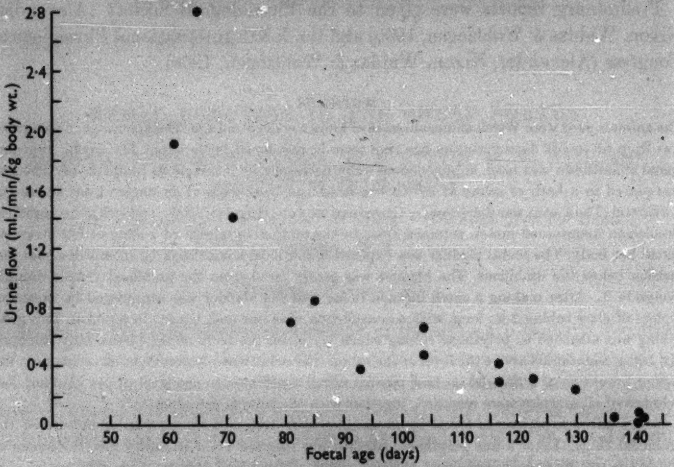

Fig. 1. Variation of urine flow per kg body weight with foetal age.

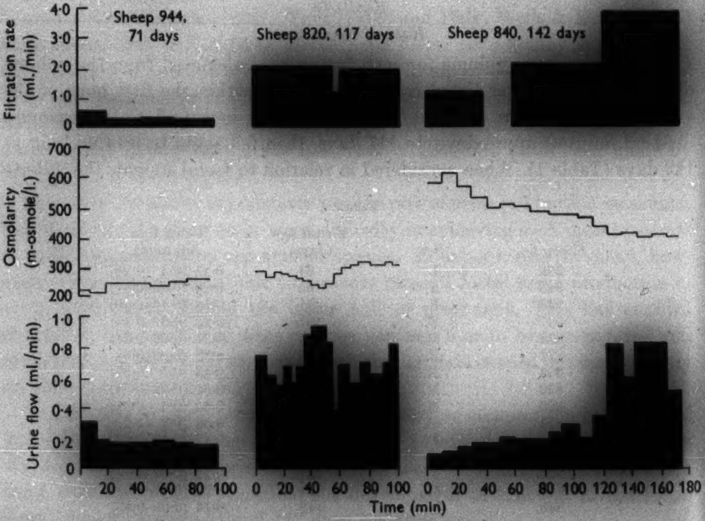

Fig. 2. Protocols of sheep aged 71, 117 and 142 days, showing variation of filtration rate and osmolarity with urine flow. Most values for filtration rate represent pooled samples covering periods of 40 min or more.

volume/kg body weight declined progressively from 1·9 ml./min/kg at 61 days to 0·04 ml./min/kg at 142 days (Fig. 1). The corresponding adult value (calculated from catheter samples on four ewes, three pregnant, one non-pregnant) was 0·02 ml./min/kg body weight.

Range of minute volume in individual foetuses

The urine flows shown in Table 1 represent the average of changing minute volumes: the extreme values encountered in each experiment are shown in brackets. Frequent variations were most marked in the 117-day foetuses, but less in the younger foetuses. The 137- to 142-day foetuses showed the capacity to produce a wide variation in urine flow but also maintained for periods of an hour or more the steadiest urine flows observed. These tendencies are illustrated in the protocols of three experiments in Fig. 2.

Composition of urine in relation to foetal age

The concentration in relation to age of the electrolytes and non-electrolytes mentioned in this paper is given in detail in the preceding paper (Alexander, *et al.* 1958). Three points are relevant to the present paper.

(1) The tonicity of the bladder urine samples described in the preceding paper was always less than that of the foetal plasma. However, during the experiments on the foetuses aged 137–142 days the urine excreted became hypertonic within an hour (about one and a half times the tonicity of the plasma) and maintained this concentration throughout the experiment.

(2) The presence of fructose in sheep foetal plasma and also, in considerable quantity, in the foetal fluids led to an investigation of its excretion by the foetal kidney.

(3) The low plasma concentration of creatinine in all but the near-term foetuses rendered it impracticable to estimate this substance on account of the limitation in volume of the blood samples which could be taken.

In view of the variable rates of urine production at different ages, and in the course of an experiment, it seemed desirable to obtain a measure of renal function by calculating the plasma clearances for various substances.

Foetal plasma clearances

Non-electrolytes. Estimation of the endogenous plasma creatinine, fructose or urea concentrations made it possible to determine the plasma clearance values for these substances by the foetal kidney. In the foetuses aged 137–142 days the plasma clearances of these three substances were very similar. In a number of the younger foetuses the quantity of blood which could be removed was limited and only the urea and fructose determinations were made. In many experiments urine samples had to be pooled to obtain sufficient for analysis. In each experiment two or more clearance values were obtained consecutively. Average values are given in Table 2. These values illustrate two points; first there is similarity of the urea and fructose clearance values in the younger foetuses as well as in the older and secondly the plasma clearance values rise with increase in gestation age.

TABLE 2

Age (days)	Urine flow (ml./min)	Plasma clearance (ml./min) Urea	Creatinine	Fructose	Mean clearance rate (ml./min/kg)
61	0·13	0·15	—	0·21	2·4
65	0·28	0·37	—	0·37	4·3
71	0·15	0·26	—	0·32	2·2
85	0·24	0·64	—	0·66	2·3
104 A	0·44	0·97	—	0·8	1·0
117 A	0·74	2·0	—	1·9	1·2
117 B	0·58	1·1	0·88	0·94	0·5
130	0·36	0·94	1·0	1·2	0·5
137	0·17	1·4	—	1·4	0·5
141	0·28	1·4	—	1·3	0·5
142	0·14	1·3	1·4	1·7	0·4

Shannon (1938) made eight determinations of the inulin clearance and exogenous creatinine clearance in an unanaesthetized adult sheep. His mean value for the inulin clearance was 58·5 ml./min. The creatinine: inulin clearance ratio was 1·03. Plasma inulin clearance estimations performed on a series of ewes (three pregnant and one non-pregnant) under spinal anaesthesia gave values similar to those of Shannon (Alexander, unpublished), and represented a clearance rate of 1·9 ml./min/kg. More recently Parry & Taylor (1955) found that the endogenous creatinine clearance in eight normal adult pregnant sheep was only 80 % of the exogenous creatinine clearance.

In the present series of experiments glomerular filtration rate in the foetus has been taken as the mean of the plasma clearance values of endogenous urea, creatinine and fructose and the percentage of filtered water reabsorbed by the foetal kidney has been calculated by use of this 'glomerular filtration rate' with the result shown in Table 3. Between 61 and 130 days gestation age the kidney absorbs one-third to two-thirds of the filtered water. After 130 days the proportion increases to 80–90 % of that filtered. This increase in the percentage of water reabsorbed coincides with an increase in the osmolarity of the urine collected. All urine samples collected from foetuses younger than 130 days gestation age showed an osmolarity lower (usually about two-thirds) than that of the foetal plasma. In the foetus aged 130 days the urine produced at the beginning and towards the end of the experiment was hypertonic, while all the other urine samples were hypotonic to varying degrees. The three foetuses aged 137–142 days produced hypertonic urine throughout the experiment (except for the initial bladder samples, which were slightly hypotonic in two foetuses). (See Fig. 2.)

Electrolyte excretion. The Na^+, K^+ and Cl^- content of all urine and plasma samples was determined, except for the plasma samples of foetuses aged 61–71 days and 137–141 days. In calculations requiring these latter values the mean of all other foetal plasma analyses was used.

The electrolyte concentrations in the foetal plasma are given in the preceding paper, as also are those of the initial bladder samples. However, it should be noted that in foetuses of the older age group (137–142 days) the urine produced subsequent to the initial bladder sample showed markedly greater electrolyte concentration. This finding suggests that while the foetus is capable of producing hypertonic urine it is not necessarily doing so *in utero*.

TABLE 3. Variation with foetal age in the percentage of water and electrolytes reabsorbed from the plasma filtrate

Foetal age (days)	Percentage reabsorbed			
	K^+	Na^+	Cl^-	H_2O
61	87	64	64	28
65	77	48	53	24
71	80	65	66	52
85	88	81	82	63
93	76	63	73	49
104 A	59	77	76	50
104 B	62	48	59	44
117 A	72	71	71	62
117 B	47	56	57	40
130	52	72	64	66
137	74	95	96	91
141	62	82	80	84
142	77	95	95	92

The amount of Na^+, K^+ and Cl filtered and reabsorbed per minute was determined for each foetus, using the 'glomerular filtration rate' as calculated above. The percentage reabsorption of these electrolytes is given in relation to age in Table 3. On the basis of the small number of observations that have been made it appears that Na^+ and Cl^- reabsorption run closely parallel, ranging from approximately 60% reabsorption at 61 days to 90% reabsorption at 142 days. In contrast the K^+ reabsorption, already 80% or more at 61 days, does not appear to increase with increase in foetal age. Comparison of these figures for electrolyte reabsorption with the corresponding values for water reabsorption (Table 3) shows that in the youngest foetuses studied the electrolyte reabsorption is significantly greater than the water reabsorption. This discrepancy accounts for the hypotonic urine observed in the young foetuses.

The foetal plasma clearance of 'total phosphate' was estimated in three foetuses. At 85 days a clearance of 0·23 ml./min was obtained as compared with a chloride clearance of 0·12 ml./min. In one foetus of 104 days the corresponding values were 0·46 ml./min for total phosphate and 0·44 ml./min for chloride, while in the second foetus of 104 days the values were 0·14 ml./min and 0·26 ml./min.

The plasma protein concentration in a number of the foetuses and ewes was calculated from the nitrogen determinations tabulated in the preceding paper. These values, ranging from 2 g/100 ml. at about 60 days to 4 g/100 ml. at 142 days, are in agreement with those obtained by Meschia (1955) for sheep foetuses of the same age range.

DISCUSSION

Davies & Davies (1950) demonstrated histologically that the mesonephros of the sheep has almost completely degenerated by the 100 mm stage. This stage represents 55 days gestation age calculated on the basis of the formula of Huggett & Widdas (1951) relating foetal length to gestation age. Davies (1950) states that his observations of the development of the metanephric arterial blood supply indicate that the metanephros may be functioning by the 40th day of gestation. The data obtained from the present experiments on sheep foetuses between 61 and 142 days of pregnancy are related therefore solely to metanephric function.

The foetal sheep metanephros has been found to produce a higher urine flow per unit body weight than the basal urine flow in the adult ewe. This high urine flow results from the early development of a high filtration rate, coincident with a low capacity for tubular water reabsorption.

In the younger foetuses the filtration rates are greater than the adult resting values when expressed per unit body weight. This high filtration rate in sheep foetuses is the reverse of that expected in the human by Gruenwald & Popper (1940) on the basis of their histological studies. To compare the filtration rates per unit body weight or per unit surface area affords little indication of the efficiency of the individual glomerulus of the foetus for two reasons. In the foetus the percentage of total body weight represented by the kidneys is roughly in inverse proportion to the foetal age. Secondly, the proportion of total kidney tissue represented by the glomeruli is greater in younger than in older foetuses.

As consideration of individual glomeruli is not possible comparison must be made of the over-all efficiency of foetal glomerular filtration with that of the adult. Ideally this should be expressed per unit of body water as suggested by McCance & Widdowson (1952) for the infant. This unit would be especially appropriate in considering development of foetal function where the percentage total body water is altering rapidly with gestation age. However, as total body water is not known in the present series of animals the unit of body weight has been taken as a rough index for comparison.

Calculation of the foetal colloid osmotic pressure on the basis of the plasma protein concentrations shows that the colloid osmotic pressure of the foetal plasma increases with gestation age. At about 60 days the oncotic pressure is estimated at 8 mm Hg. By 141 days this has increased to 18 mm Hg as compared with 25 mm Hg in the adult ewe.

In two of the older foetuses a continuous recording of foetal blood pressure throughout the experiment was made by a cannula passed via a tributary into the lumen of one of the umbilical arteries. The mean blood pressure thus recorded by capacitance manometer was 45 mm Hg. The adult ewe's femoral arterial pressure averaged 123 mm Hg. The difference between the arterial blood pressure and the pressure due to the plasma proteins is smaller in the near-term foetus than in the adult ewe and this may account for the nearly fourfold difference in the corresponding filtration rates.

In the course of each experiment variations in the filtration rate were observed but these were greatest in foetuses near term (see Fig. 2). These variations, which were reflected in the urine flow, indicate some instability in the control of the glomerular filtration pressure which was not reflected in the umbilical blood-pressure recording.

In the present paper foetal renal tubular function has been considered only with respect to the development of water and electrolyte reabsorption. At 65 days, when the filtration rate per unit body weight was the highest measured, the proportion of water reabsorbed is only 25%. There is a steady increase with foetal age from 65 days to 140 days, when 80% or more is reabsorbed. During the period 61–130 days sodium and chloride reabsorption is of the order of 60–80% and the potassium reabsorption slightly higher. From 130 to 140 days sodium and chloride reabsorption rises to approximately 95% and potassium is of the order 60–80%. The combined effect of these different rates of water and electrolyte reabsorption results in the formation of hypotonic urine between 61 and 130 days gestation age and subsequently of hypertonic urine between 130 and 142 days gestation age.

Thus, early in development the metanephric tubules are capable of osmotic work in the sense of active electrolyte reabsorption, while it is only late in gestation that they begin to develop the adult capacity for active water reabsorption and production of a hypertonic urine.

SUMMARY

1. The urine production of fourteen sheep foetuses between 61 and 142 days gestation age has been measured.

2. The rate of urine flow was maximal at 117 days. It then declined towards term. Expressed on the basis of flow per unit body weight the urine production of even the most advanced foetus was observed to be in excess of adult resting values.

3. The glomerular filtration rate per unit body weight was greater than the adult value at 61 days, but by 142 days it had decreased to about one quarter of the adult value.

4. Electrolyte (Na^+, K^+, Cl^-) reabsorption occurs in excess of water reabsorption between 61 and 130 days, resulting in the production of hypotonic urine.

5. Tubular water reabsorption does not exceed 66% until 130 days: 80–90% reabsorption occurs between 130 and 142 days, coinciding with the production of hypertonic urine.

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EFFECTS OF ADRENALECTOMY ON OXYGEN POISONING IN THE RAT

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Until recent years little work seems to have been done to find out whether or not the adrenal glands are involved in the disturbances arising from exposure to oxygen under pressure. This seems all the more remarkable in view of the volume of work on 'stress' and the importance of the adrenal glands in determining the reactions of the body to it. Campbell (1937) increased the severity of toxic manifestations in rats by administering adrenaline. Bean (1951) discovered that repeated exposure of rats to oxygen under high pressure resulted in hypertrophy of the adrenal cortex. Brief reports of the earlier experiments in this paper have been given to the International Physiological Congress in Montreal, and to the Physiological Society (Taylor, 1953, 1954). While the work described below was in progress, both Bean and Gerschman and their colleagues published a considerable number of interesting papers on the subject; these are dealt with in the discussion.

METHODS

Animals and diet. Hooded rats of the Rowett strain, from a colony kept in this laboratory and closed for some forty generations, were used in all experiments but two. In these two the reactions of this strain were compared with those of the eighth generation in descent from a wild black × albino mating. All animals were fed throughout on the stock diet which differs from that of Thomson (1936) only in that it contains 14%, not 7%, of skim milk. The animals were aged 3–5 months, and in any given experiment (apart from Expts. 1 and 7) they were of the same age. In Expt. 1 there was a considerable variation in age and Expt. 7 was designed to test the effect of age. In the light of later experiments the age variation may have had a slight influence on the results in Expt. 1, but insufficient to affect the general conclusions.

Operation and post-operative maintenance. Bilateral adrenalectomy was carried out, under ether anaesthesia, through a central dorsal skin incision. From the day of operation the rats were given 0·9% NaCl solution to drink, except in Expt. 8. In other respects their maintenance did not differ from that of unoperated control animals.

Exposure to oxygen under pressure. The details of exposure have been described (Taylor, 1956). Briefly, the animals were placed in the steel chamber which was then flushed with some 400 l. O_2. Pressure was then raised at a uniform rate to 90 Lb./sq. in. (6 atm approx.). CO_2 was absorbed by trays of soda-lime. Room temperature was kept, as far as possible, at 20° C.

In some experiments adrenalectomized animals were exposed twice. They were exposed first for a fixed period of time (50 min) at the end of which all those showing any evidence of O_2 poisoning were rejected. The others were then divided into treatment groups and re-exposed at a later date.

Drugs. Thyroid powder (Armour Laboratories) was administered daily by mouth (10 mg/100 g body wt./day) as a suspension in water. Deoxycortone acetate B.P. (DOCA) and cortisone acetate (Merck and Co.) were each injected intramuscularly twice daily in doses of 0·5 mg/100 g body wt. Hydrocortisone acetate (Roussel Laboratories) was similarly injected twice daily in doses of 0·05 or 0·5 mg/100 g body wt. Cortin (Eucortone, Allen and Hanbury), which is the whole cortical extract, was administered subcutaneously in two daily doses of 0·5 ml./100 g body wt. Adrenaline (0·01 mg/100 g body wt). was given subcutaneously as the hydrochloride in a single dose 15 min before exposure.

Duration of treatment, and also the period of time between operation and exposure, varied somewhat from experiment to experiment.

Assessment of results. The method of assessing general effects has been described already (Taylor, 1956). Severe poisoning is usually indicated by convulsions, but sometimes prostration occurs without motor disturbance. This prostration is often, but not always, accompanied by hyperpnoea. The interval from the initial raising of the O_2 pressure to the appearance of such prostration or convulsions, whichever was earlier, was taken as the 'time to the onset of severe poisoning'.

In some operated animals neither convulsions nor prostration occurred after exposures varying from 70 to 120 min, the experiments being stopped in most cases when respiratory distress became apparent, and in a very few instances, merely on account of the passage of time. In all these cases, to permit of statistical assessment of the results, the time to the beginning of decompression was taken as the 'time to onset of severe poisoning'.

Post-mortem examination was carried out on all animals. Lung damage was assessed on an arbitrary scale of 0–5. In Expt. 2, which involved much the largest number of rats, a careful histological examination was made of tissues in the field of operation, in an attempt to discover either aberrant cortical tissue or evidence of incomplete removal of a gland. Specimens were preserved in 'Susa' (Heidenhain), blocked in ester-wax (British Drug Houses) and stained with haematoxylin-eosin (Carleton & Leach, 1947).

RESULTS

Experiment 1. Two groups of sixteen rats, three male and thirteen female in each, were used. One group was adrenalectomized and the other retained as a control. Two of the operated animals died before exposure, which took place on the fourteenth day after operation.

All the sixteen controls had severe convulsions between 26 and 44 min after exposure began; the mean time was 36·2 ± 1·5 min (S.E. of mean). Six of the fourteen operated animals became convulsed, and for the remainder exposure ended with the onset of respiratory distress. The duration of exposure ranged from 45 to 90 min, except for one rat which had an anomalous convulsion at 16 min; this last value was omitted, the mean time for the others being 68·5 ± 3·9 min. The time was more variable for the operated group, but the evidence for a longer tolerance by operated animals was overwhelming. Lung damage in the two groups could not be compared because the control rats were saved for further tests.

Eighteen days later, fourteen of the erstwhile control rats were divided into two groups of seven, of which one group was adrenalectomized. After 14 days (i.e. 4–5 weeks after their first exposure) all were exposed again and the time to onset of severe poisoning recorded. Table 1 shows the mean times for first and second exposures. The unoperated animals had essentially the same time on both occasions, but adrenalectomy increased the time for the other group very substantially, bringing it to about the same level as for the other animals, operated on in the first part of the experiment. Indices of lung damage were obtained for the fourteen animals; the difference between the means of 3·14 ± 0·37 for the unoperated and 1·86 ± 0·37 for the operated was statistically significant.

Table 1. Expt. 1. Mean times (min) to the onset of severe poisoning in rats exposed to oxygen at a pressure of 6 atm. The rats in Part 2 comprise fourteen of the controls used in Part 1

Part 1

Control	Adrenalectomized	Difference
36·2	68·5	32·3±4·2*

Part 2

	Control throughout	Adrenalectomized after 1st test	Difference
1st test	35·7	34·1	−1·6±2·0*
2nd test	37·1	65·3	28·2±5·5

* s.e. of mean.

Experiment 2. In this experiment adrenalectomized and control rats were compared in respect of the effects of different drugs; animals received 'no drug', cortisone, DOCA or thyroid, and were independently assigned to 'adrenaline' or 'no adrenaline' groups. Equal numbers of male and female rats were used in a balanced manner. On account of the evidence for greater variation in sensitivity amongst adrenalectomized rats shown in Expt. 1, twice as many rats were allotted to the operated group as to the unoperated. In all, 144 rats were used in a 4×2^3 factorial scheme, having threefold replication of unoperated and sixfold replication of operated animals. In order to eliminate any trends with time, 'blocks' of twelve rats were used on each of twelve occasions in accordance with a design confounding each complete replicate of the 4×2^3 scheme into quarters and losing information on three unimportant interactions. Each block of twelve contained four unoperated animals, two of each sex, two with and two without adrenaline, and one on each of the other four drug treatments; it also contained eight operated animals, twice as many from each category.

Exposure to oxygen took place 2 weeks after adrenalectomy, treatments being started on the eighth post-operative day and continuing to include the day of exposure. Ages of all animals on their day of exposure were comparable, and the whole experiment extended over 12 weeks.

Seven rats died, and appropriate adjustments to the statistical analyses were used to take account of this. A few of the times to onset of poisoning were recorded simply as *lower* limits (75+, 100+, etc.), and these were all arbitrarily taken as 100. The mean times to onset of severe poisoning are given in Table 2. Despite the precautions taken in designing the experiment, the variability in the response to high tension of oxygen was as high as in Expt. 1; there was no correlation between susceptibility of rats and their changes in weight.

The operated animals again had significantly longer times to onset of severe poisoning, though the difference was smaller than in Expt. 1. The average time for females was 7·5 min longer than for males, also a statistically significant difference. No large effects of the drugs appeared, though there was some suggestion that cortisone increased and thyroid decreased the time to onset; adrenaline appeared to decrease the time somewhat in the absence of other drugs or in the presence of cortisone, but to increase it in the presence of DOCA. None of these drug effects was clearly significant.

TABLE 2. Expt. 2. Mean times (min) to the onset of severe poisoning in rats exposed to oxygen at a pressure of 6 atm, and treated with various drugs

	Control	Adrenalectomized	Difference
Male	35·1	48·7	13·6±4·0*
Female	41·5	57·4	15·9±4·0
Mean	38·3	53·0	14·7±2·8

	No drug	Cortisone	DOCA	Thyroid	Mean (±2·0)
No adrenaline	48·7	55·2	41·0	42·1	46·8
Adrenaline	41·3	47·3	47·4	42·3	44·6
Mean (±2·8)	45·0	51·2	44·2	42·2	45·7
Difference (±5·6)	−7·4	−7·9	6·4	0·2	−2·2±2·8*

Unoperated rats only

	No drug	Cortisone	DOCA	Thyroid	Mean (±1·8)
No adrenaline	39·3	42·5	40·7	29·8	38·1
Adrenaline	34·0	40·0	47·0	33·0	38·5
Mean (±2·6)	36·7	41·2	43·8	31·4	38·3
Difference (±5·2)	−5·3	−2·5	6·3	3·2	0·4±2·6*

* ±S.E. of mean.

Table 3 summarizes the records of lung damage. The reduction in damage in the adrenalectomized animals was highly significant, the sex difference being negligible. DOCA and thyroid both significantly increased the lung damage, irrespective of whether or not the animals were adrenalectomized, but cortisone and adrenaline appeared to be practically without effect.

In twenty-one animals tissue was present which microscopic examination showed closely to resemble suprarenal cortical tissue. A typical example is illustrated in Pl. 1*b* and compared with normal rat tissue in Pl. 1*a*. The times to the onset of severe poisoning in these twenty-one rats were apparently distributed at random over the whole range; four gave values of 90 min or more.

TABLE 3. Expt. 2. Mean index of lung damage in rats exposed to oxygen at a pressure of 6 atm and treated with various drugs

	Control	Adrenalectomized	Difference
Male	3·75	2·65	−1·10±0·29*
Female	3·67	2·25	−1·42±0·29
Mean	3·71	2·45	−1·26±0·21

No drug	Cortisone	DOCA	Thyroid	
2·71	2·73	3·60	3·27	(±0·21)

* ±S.E. of mean.

Experiment 3. As already stated, despite special precautions in the previous experiment the variability remained high, and it was thought that the effects of drugs, particularly of DOCA, might therefore be masked. As a further test, thirty-two rats, sixteen male and sixteen female, were adrenalectomized and, on the fourteenth post-operative day, were exposed for 50 min to oxygen at 6 atm. All showing any evidence of poisoning were discarded, and the others were put at random into two groups, each of four males and three females. One group was given injections of DOCA for 8 days, the other was untreated. Fourteen days after the initial exposure, both groups were again exposed to oxygen at 5 atm, except for one animal of the control group which had died.

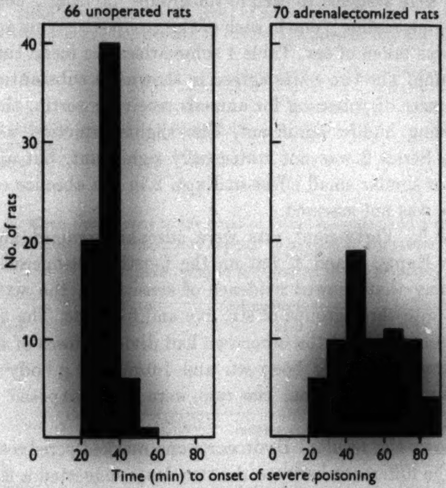

Fig. 1. Block diagram showing distribution of times to the onset of severe poisoning in all adrenalectomized and unoperated rats, exposed for the first time and given no drug treatment.

The times to onset of severe poisoning were on average almost equal, 52·7 ± 6·7 min and 55·0 ± 6·2 min for the control and DOCA groups respectively. Contrary to the findings of Expt. 2, the lung damage also was almost the same in the two groups, 2·43 ± 0·57 and 2·50 ± 0·62 respectively; however, with standard errors as large as these, an effect on lung damage could escape detection.

Experiment 4. Two series of adrenalectomized rats were used, each consisting of the survivors of thirty-two rats exposed in precisely the same way as in the preliminary part of Expt. 3. The two series were tested on different occasions, but for both experimental conditions were as far as possible the same as in Expt. 3 and the results are therefore presented together.

Series 1 consisted of fourteen rats, seven of which were given no drug and seven 'cortin'; one of the control group died. Series 2 consisted of twenty rats, of which five were assigned to each of the groups 'no drug', 'cortin', 'adrenaline', 'cortin + adrenaline'; one in each of the groups receiving adrenaline died. No account was taken of sex. Table 4 summarizes the mean times to onset of severe poisoning. The two series agreed in showing a substantial reduction in time to the onset of poisoning for animals receiving cortin, the difference of 19 ± 4 min being highly significant. The slight reduction associated with adrenaline in Series 2 was not statistically significant, but agrees with the indication of a similar small effect in Expt. 2 in the absence of other drugs. Lung damage was not assessed.

Experiment 5. Forty-eight rats were adrenalectomized and exposed to oxygen as in Expts. 3 and 4, but on the fourth post-operative day. After rejection of any that showed evidence of sensitivity, the sixteen survivors were divided into three groups of six, five and five rats. The first group was retained as a control, and the other two had divided doses of hydrocortisone for 5 days at 0·1 mg/100 g body wt. and 1·0 mg/100 g body wt. daily. On the ninth day after operation, the rats were again exposed to oxygen at 6 atm.

Table 5 shows the results. Hydrocortisone markedly reduced the time to convulsion; the difference between the two doses suggested a lesser reduction at the higher dose, but this was not significantly different from the lower dose. Lung damage appeared to be rather greater in the rats that received hydrocortisone, but on these few animals the effect was not sufficiently great to be regarded as clearly demonstrated.

Experiment 6. Richter & Uhlenhuth (1954) have suggested that, under repeated inbreeding, laboratory rats become progressively less dependent on the suprarenal cortical tissue than are wild rats, and that the amount of this tissue is much less than in the wild strain. If this were so, the variety in sensitivity observed in the operated animals in this series of experiments might be due, in part, to the strain of rats. In order to examine this point, a group of twelve male black rats, of the strain described earlier, was compared with a similar group of hooded rats. Six of each group were adrenalectomized and, 6 days later, all were exposed to oxygen at 6 atm.

The difference in weight of suprarenal tissue in the two strains was very great, averaging 13·2 mg/100 g body wt. for the hooded and 20·1 mg/100 g body wt. for the black; the standard error of each mean was 0·5 mg/100 g; so that the difference was highly significant. The hooded rats were slightly heavier, averaging 415 g against 380 g for the black. Nevertheless, as Table 6 shows, the strains scarcely differed in their reactions to adrenalectomy. The hooded gave rather shorter times to onset of poisoning and rather greater lung damage than the black, whether or not they were adrenalectomized, but differences between operated and control groups were very similar for the two strains. The experiment involved too few rats to detect small differences between strains, but there was certainly no evidence of any large difference.

TABLE 4. Expt. 4. Mean times (min) to the onset of severe poisoning in adrenalectomized rats exposed to oxygen at a pressure of 6 atm and treated with various drugs

	No cortin	Cortin	Difference
Series 1	71·0	46·3	−24·7±6·0*
Series 2, no adrenaline	60·8	43·2	−17·6±6·8
Series 2, adrenaline	51·0	38·5	−12·5±7·6
Weighted mean difference	—	—	−19·2±3·9

* ±S.E. of mean.

TABLE 5. Expt. 5. Mean times (min) to the onset of severe poisoning and mean index of lung damage in adrenalectomized rats exposed to oxygen at a pressure of 6 atm and treated with hydrocortisone

	Daily dose of hydrocortisone		
	None	0·1 mg/100 g	1·0 mg/100 g
Mean time (min) to onset of severe poisoning	55·8±3·7*	33·0±4·0	42·8±4·0
Lung damage	1·0±0·6	2·4±0·6	2·2±0·6

* ±S.E. of mean.

TABLE 6. Expt. 6. Mean times (min) to the onset of severe poisoning and mean index of lung damage in hooded and black rats exposed to oxygen at a pressure of 6 atm

	Time (min) to the onset of severe poisoning		
	Control	Adrenalectomized	Difference
Hooded	32·7	41·5	8·8±6·1*
Black	37·5	48·2	10·7±6·1
Mean	35·1	44·8	9·7±4·3

	Lung damage		
	Control	Adrenalectomized	Difference
Hooded	3·7	2·5	1·2±0·79
Black	1·7	1·3	0·4±0·79
Mean	2·7	1·9	0·8±0·56

* ±S.E. of mean.

Experiment 7. Hartman & Brownell (1949) have stated that accessory cortical tissue occurs in a high proportion of young rats. If the variability of previous experiments were due to the presence of functioning accessory tissue, young animals might fail to show increased resistance after adrenalectomy more frequently than old.

This hypothesis was examined by exposing seven groups of eight rats, four males and four females in each, to oxygen at 6 atm. The groups were aged 1, 2, 3, . . ., 7 months. Two males and two females in each group were adrenalectomized. Three rats died.

TABLE 7. Expt. 7. Effect of age on mean time (min) to the onset of severe poisoning in rats exposed to oxygen at a pressure of 6 atm

	Age of rats (months)							
	1	2	3	4	5	6	7	S.E.
Control	28	29	29	32	34	37	40	±2·0
Adrenalectomized	32	49	45	48	58	60	58	±8·0
Difference	4	20	16	16	24	23	18	±8·2

TABLE 8. Expt. 7. Effect of age and of sex on mean index of lung damage in rats exposed to oxygen at a pressure of 6 atm

	Age of rats (months)							
	1	2	3	4	5	6	7	S.E.
Control	2·5	3·0	1·8	3·2	3·0	2·0	1·5	±0·63
Adrenalectomized	2·0	0·5	0·5	2·8	2·0	1·8	2·2	±0·63

	Control	Adrenalectomized	Mean (±0·24)
Male	3·14	2·14	2·64
Female	1·71	1·21	1·46
Mean (±0·24)	2·43	1·68	2·05

As usual, the times to onset of severe poisoning were consistently longer and much more variable for the adrenalectomized rats. Table 7 shows the mean times. The unoperated rats gave evidence of a small but steady increase in time with increasing age, amounting to about 2·0 ± 0·4 min/month; both the times and the rate of increase were rather greater for females than for males. The operated rats showed a much greater rate of increase, 3·9 ± 1·5 min/month, but the greater variability prevents any certainty that this rate differs from the other; there was no sign of any sex difference. Although by no means conclusive, the results of the experiment are in accordance with the hypothesis under test. At 1 month of age the difference between adrenalectomized and normal rats in respect of tolerance of oxygen at high pressure appears to be small, but it increases quite markedly at any rate up to 7 months.

There was no evidence that age affected lung damage (Table 8), but damage was significantly less for the adrenalectomized rats and significantly less for females than for males.

Experiment 8. If repeated exposure of rats to oxygen under high pressure were to lead to suprarenal cortical hypertrophy, then such hypertrophy should affect any accessory cortical tissue and repeated exposure might reasonably be expected to lead to an increased sensitivity to oxygen. Moreover, if post-operative maintenance on 0·9% NaCl solution were to give more protection against the full development of cortical insufficiency, thus tending to increase sensitivity to oxygen, then maintenance of the animals on water might have the opposite effect. Expt. 8 was planned as a test of these possibilities.

TABLE 9. Expt. 8. Mean times (min) to the onset of severe poisoning in control and saline- and water-maintained, adrenalectomized rats on single or after repeated exposure to oxygen at a pressure of 6 atm

No. of exposures	Control	Adrenalectomized With H_2O	Adrenalectomized With 0·9% NaCl solution	Mean ±S.E.
Single	31·3	51·5	58·7	47·2±3·7
Repeated	32·7	42·5	44·2	39·8±3·7
Mean±S.E.	32·0±4·3	47·0±5·2	51·4±4·3	43·5

Six litters of rats (four of black and two of hooded) were used, each consisting of three males and three females. Four of each litter were adrenalectomized, two being thereafter maintained on 0·9% NaCl solution and two on water; the other two were kept as unoperated controls. One animal from each of these pairs was exposed to oxygen at 6 atm for 20 min on 5 of the 7 days preceding final exposure on the fifteenth post-operative day. Four rats, all adrenalectomized and maintained on water, died before the final exposure. Of the eight other rats that were adrenalectomized and maintained on water, seven lost weight between operation and final exposure; on the other hand, of the twelve adrenalectomized rats maintained on NaCl solution, ten gained in weight between operation and final exposure.

Adrenalectomy again increased the time to onset of severe poisoning, to an extent similar to that of other experiments (Table 9). There was little difference between the operated rats maintained on water and on NaCl solution. The control rats showed the same time to onset, irrespective of whether or not they had had repeated exposure before the final tests; the adrenalectomized showed somewhat shorter times if they had been exposed repeatedly, though the difference from the unexposed was not statistically significant. Neither sex nor strain of rat appeared to modify these effects. There was no correlation between change in weight of rats and susceptibility to oxygen.

Table 10 summarizes results in respect of lung damage. The adrenalectomized rats had less damage, but variability from animal to animal was high and the difference from the controls was not statistically significant. No consistent difference between water and NaCl appeared, nor was there any effect of repeated exposure. Indeed, the only clear result from the lung damage records was the sex difference, 2·1 for males and 1·2 for females with a standard error of ±0·3 for each.

Distribution of times to the onset of severe poisoning in unoperated and adrenalectomized rats. The figures for time to onset of severe poisoning for all rats in Expts. 1–8, irrespective of age, strain or sex, but excluding those given any drug treatment, and those not being exposed for the first time, have been used to form a block diagram (Text-fig. 1).

Table 10. Expt. 8. Mean index of lung damage in control and saline- and water-maintained adrenalectomized rats on single or after repeated exposure to oxygen at a pressure of 6 atm

No. of exposures	Control	Adrenalectomized		Mean ±S.E.
		With H_2O	With 0·9% NaCl solution	
Single	2·5	1·2	1·5	1·7±0·3
Repeated	1·8	1·7	1·2	1·6±0·3
Mean ±S.E.	2·2±0·4	1·4±0·5	1·3±0·4	1·6

It is easily seen that of the sixty-six unoperated rats the overwhelming majority gave values lying between 20 and 40 min. In the case of the seventy operated animals the values lie between 20 and 90 min, half of them being between 20 and 50 min, but distributed more widely than are the controls; half are similarly distributed between 50 and 90 min. A very few of the experiments in this latter group were terminated, as explained earlier, before unequivocal manifestations of toxicity were observed. Longer exposures in these cases would have extended the graph still farther to the right, while lowering its peak, and there was no definite evidence of a bimodal distribution.

DISCUSSION

The experiments which have been described show clearly that unanaesthetized, adrenalectomized rats are more resistant to the effects of oxygen under pressure than are unoperated controls. This result was obtained independently by Bean, Johnson & Smith (1953) and by Gerschman & Nadig (1953).

The increase in resistance was much less obvious in terms of lung damage than in the case of the central nervous system effects, and there was no correlation between the two types of effect. Cardiac and respiratory slowing are known to occur in animals exposed to oxygen under high pressure. Unpublished experiments by the author have shown that anaesthetized, adrenalectomized rats are just as liable to the cardiac and respiratory slowing produced by oxygen as are normal animals. The two groups therefore seem to differ mainly in their responses to the action of oxygen under pressure on the central nervous system, the difference between operated and unoperated rats in this respect being very great, despite the variability in the responses of the former.

If one is to accept the existence of a 'general adaptation syndrome' as postulated by Selye (1949), one is forced to conclude that, exposed to this particular 'stress', adrenalectomized animals behave in a way opposite to that in which they behave when exposed to more orthodox 'stressor agents'. Indeed, Gerschman & Fenn (1954) have suggested that oxygen at high pressure is an 'unnatural stress', and that therefore the ordinary rules may not apply. It seems better therefore to leave aside the whole vexed question of 'stress' and to attempt an explanation in terms of factors already known to influence the course of oxygen poisoning.

Bergen, Hunt & Hoagland (1953) and Hoagland (1955) showed that the rate of cerebral blood flow in adrenalectomized, saline-maintained rats was considerably less than in normal rats. There was also a reduction of 18% in brain oxygen consumption, and these changes were accompanied by a reduction in the frequency of the electrocorticogram. The probable importance in oxygen poisoning of any factor which reduces oxygen utilization at the cellular level has been discussed (Taylor, 1956). Lambertsen, Kough, Cooper, Emmel, Loeschchke & Schmidt (1953) found a decrease in cerebral blood flow in human subjects exposed to oxygen at high pressure, and suggested that this, by lowering the average oxygen pressure at which the tissues of the brain were functioning, would have a protective effect. If such a decrease in cerebral blood flow were exaggerated by adrenalectomy, this by itself might go far in explaining the protective effect of the operation.

Given that adrenalectomy had a definite protective effect, it seemed probable that replacement therapy by one or other of the substances in common use might reverse the effect, and in so doing perhaps point to a possible cause. The variation in the responses of the operated animals made it necessary, after Expt. 2, to use animals which on previous exposure had been shown to be resistant to the effects of oxygen.

DOCA was not effective in reversing the protection given to the central nervous system by adrenalectomy; and this, together with the absence of any significant difference between saline-treated and water-treated animals in Expt. 8, seems to show that alterations in water and electrolyte metabolism resulting from the operation are not responsible for the difference between operated and unoperated animals. Cortisone did not significantly alter the sensitivity of either group. In fact some of the highest times to onset of severe poisoning were recorded in cortisone-treated, adrenalectomized animals. Bergen *et al.* (1953) and Hoagland (1955) showed that the cerebral, circulatory and metabolic changes occurring after adrenalectomy were abolished by administration of whole cortical extract or cortisone, but not of DOCA. The increase in sensitivity to oxygen brought about by cortin, and the absence of any such increase with DOCA, would support the view that such changes were responsible for the increased resistance of adrenalectomized animals, while the results obtained with cortisone are a stumbling-block. Gerschman, Gilbert, Nye, Nadig & Fenn (1954) found that cortisone in small doses increased sensitivity to oxygen significantly in adrenalectomized rats only, but in large doses had the opposite effect, decreasing sensitivity in both operated and control groups. The dose of cortisone used in Expt. 2 corresponded to the large dose of Gerschman and her colleagues, and this might possibly explain some of the results. On the other hand, cortisone had no effect whatsoever on control rats. It is interesting, too, that in Expt. 5 both large and small doses of hydrocortisone had the effect of reducing tolerance in adrenalectomized rats. According to Vogt (1954) the differences between the actions of hydrocortisone and cortisone are only quantitative, hydrocortisone being more active in most respects; of the two, the rat secretes mainly cortisone. It is strange therefore that cortisone failed to show any definite effect in Expt. 2.

Bean & Johnson (1954) found that adrenaline augmented the adverse action of oxygen at high pressure, confirming the work of Campbell (1937). Gerschman *et al.* (1954) and Gerschman, Gilbert, Nye, Price & Fenn (1955) thought that adrenaline had some effect but concluded that the adrenal cortex played a more important role in oxygen poisoning than did the medulla; this is supported by the results of Expts. 2 and 4.

There was a suggestion that thyroid in Expt. 2 decreased the time to onset of severe poisoning in unoperated animals. Campbell (1937), Gersh & Wagner (1944) and Grossman & Penrod (1949) showed that thyroid had such an effect. On the other hand, thyroid had no significant effect on operated animals and some of the times to onset of severe poisoning were amongst the longest recorded. The general opinion is that administration of thyroid increases the requirements for cortical hormone (Pincus & Thimann, 1955). Presumably any tendency to deficiency could be counteracted in an intact animal, but obviously not after adrenalectomy. In the first case thyroid could exert an independent effect, increasing sensitivity to oxygen under pressure, while after adrenalectomy any effect could well be masked by the degree of protection given by the loss of the adrenals. It is worth noting that administration of thyroid aggravated the effects of adrenalectomy before exposure to oxygen since, in Expt. 2, all deaths but one before exposure to oxygen were in thyroid-treated operated rats.

One of the most striking features of these experiments is the wide range of values for the time to onset of severe poisoning in the adrenalectomized animals as a group. This made it difficult to draw conclusions even when relatively large numbers were used.

The most likely cause is failure to remove all the cortical tissue in the body, with subsequent hypertrophy of cortical 'rests', not necessarily in the perirenal area. Hartman & Brownell (1949, quoting Jaffe) state that macroscopic amounts of accessory cortical tissue occur in 8 % of normal rats and in 20–25 % after removal of the suprarenal glands, and that microscopic accessories have been detected in 70 % of immature rats. In Expt. 2 histological examination showed that tissue resembling suprarenal cortical tissue was present in about one quarter of the operated animals, but the values for the time to onset of severe poisoning could not be correlated with the presence of such tissue, and indeed four out of the twenty-one animals concerned had very high values.

The whole situation is probably so complicated that these results cannot be held to dispose of the hypothesis that adrenal cortical rests are in fact responsible for the variation in tolerance, especially when one remembers the difficulty involved in identifying such tissue at the site of operation and the impracticability of determining just how much may be present at other sites in the body, quite apart from its functional status. But equally, much more experimentation is required before the hypothesis can be finally accepted.

SUMMARY

1. The effects of adrenalectomy and of substitution therapy on the manifestations of oxygen poisoning were studied in rats of two strains, exposed to a pressure of 6 atm of pure oxygen.

2. Adrenalectomy gave very definite protection against the central nervous system manifestations of oxygen poisoning, and gave some protection against lung damage.

3. The variability in time of appearance of nervous system effects in adrenalectomized animals was very great. This variability was not confined to either one of the two strains tested, although these possessed suprarenal glands that differed considerably in weight. It was not correlated with the presence of small amounts of residual cortical tissue in the perirenal region. It may possibly depend on accessory cortical tissue elsewhere in the body.

4. There was an increase with age in the time to onset of severe poisoning; this was greater in adrenalectomized than in unoperated animals, but the greater variability in the former made it impossible to say that this represented a real difference.

5. Of the drugs tested, cortin and hydrocortisone were capable of increasing susceptibility of operated animals to the effects of oxygen on the central nervous system. Cortisone, DOCA and thyroid had no significant effect. There was a possibility that adrenaline slightly increased susceptibility.

6. There was no significant difference between the responses of adrenalectomized animals maintained on 0·9% saline and those maintained on water. In general there was no relation between the time to onset of severe poisoning and gain or loss in weight.

7. Possible explanations for the difference between adrenalectomized and unoperated animals are discussed.

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EXPLANATION OF PLATE

Plate 1. (*a*) Normal adrenal cortical tissue from rat; (*b*) tissue removed from perirenal region of rat whose adrenals had been excised 14 days previously.

THE JOURNAL OF PHYSIOLOGY, Vol. 140, No. 1 PLATE 1

a
b
c
d
0·1 mm
0·1 mm

(*Facing p.* 36)

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EFFECTS OF TOCOPHEROLS, METHYLENE BLUE AND GLUTATHIONE ON THE MANIFESTATIONS OF OXYGEN POISONING IN VITAMIN E-DEFICIENT RATS

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Previous work (Taylor, 1953; Gerschman, Gilbert, Nye & Fenn, 1955) has shown that rats deficient in vitamin E are more susceptible to the effects of oxygen under pressure on the central nervous system and on the lungs than are tocopherol-treated rats. Such deficient animals also suffer a considerable degree of haemolysis *in vivo* when exposed to oxygen, but this is completely prevented by tocopherol (Taylor, 1956). Methylene blue gives some degree of protection against the effects on the nervous system, although it has no influence on either lung damage or haemolysis (Taylor, 1956).

This paper describes investigations into the effects of α- and γ-tocopherol, methylene blue and glutathione on rats exposed to oxygen for various periods of time and at various pressures.

METHODS

Animals and diet. Hooded rats of the Rowett strain were used. All litters were weaned on the 23rd day and placed on the same basic vitamin E-deficient diet (Taylor, 1956). The salt mixture was no. 351 of Hubbel, Mendel & Wakeman (1937). Vitamin A palmitate and calciferol were given orally by pipette, twice weekly, in olive oil in doses of 100 and 60 i.u. respectively, and tocopherols when given were added to this mixture. Methylene blue was given mixed with the diet (150 mg/100 g). Glutathione (2 mg/g body wt.) was injected subcutaneously in a single dose 30 min before exposure to oxygen. The reduced form (L. Light and Co.) was used, dissolved in water and with pH adjusted to 6·0 by means of 10 % NaOH.

Exposure to oxygen under pressure. Conditions of exposure were standardized as far as possible (Taylor, 1956). In Expt. 1 the pressure was raised to 75 Lb/sq.in. (approx. 5 atm) while in Expt. 2 pressures of 55, 75, 95 and 115 Lb./sq.in. were used—i.e. a range of from just under 4 to just under 8 atm.

Assessment of general effects. The manifestations of oxygen poisoning in unanaesthetized rats so exposed have already been described (Taylor, 1956). In some of the experiments involving vitamin E-deficient animals it was extremely difficult to gauge the time to onset of severe poisoning, since they often succumbed rather gradually without any observable motor disturbances; only when repeated tapping on the wall of the chamber failed to elicit any movement was it decided that the animal was severely prostrated. The times in such cases are therefore not accurate to more than about ±5 min, a point of some importance in Expt. 2, where at the higher pressures the times for all groups of animals are rather short. Lung damage was assessed at autopsy as described in the earlier paper (Taylor, 1956).

Assessment of vitamin E deficiency. In all males the testes were weighed for evidence of atrophy, and in all females the uterus was examined for the chestnut brown pigmentation characteristic of vitamin E deficiency. The dialuric acid haemolysis test (Rose & György, 1950) was carried out on blood from all animals, before exposure. The animals were lightly anaesthetized with ether and samples taken from the tail.

Haemolysis in vitro and in vivo. Blood sampled before exposure was added to 20 vol. of NaCl solution 0·9% (w/v), and blood taken after decompression was added to 20 vol. of the saline-citrate solution as in the dialuric acid test. In Expt. 1 the tubes containing blood drawn before exposure were exposed *in vitro* with the animals from which the blood was taken. In Expt. 2 such samples were taken before exposure only from those rats to be exposed to 75 Lb./sq.in. O_2. These samples were stirred by means of a small paddle driven by a clockwork motor, and were placed in a water-bath originally at 40° C, which cooled during exposure to room temperature. All samples exposed *in vitro* and *in vivo* were finally centrifuged and the colour of the supernatant fluid was compared, in a Spekker photoabsorptiometer using an Ilford 'spectral blue' filter (no. 602), with the colour obtained when blood samples were completely haemolysed in 20 vol. of distilled water. In Expt. 2 additional blood samples, taken before exposure and suspended in saline-citrate solution, were centrifuged and allowed to stand for 24 hr under ordinary atmospheric conditions.

Design of experiments

Experiment 1. This experiment was designed to investigate the effects of duration of exposure to oxygen under high pressure, and of eight different treatments, on the degree of haemolysis *in vivo* and on survival. The times of exposure were 15, 30, 45 and 60 min. The treatments, apart from a control group, consisted of additions to the basic diet and were as follows: T_1, no addition (control); T_2, α-tocopherol acetate (20 mg/week); T_3, α-tocopherol acetate (1 mg/week); T_4, α-tocopherol acetate (25 mg in 1 dose on day before exposure); T_5, γ-tocopherol (20 mg/week); T_6, γ-tocopherol (1 mg/week); T_7, glutathione (2 mg/g body wt.); T_8, methylene blue (0·15 g/100 g diet). The animals were maintained thus, after weaning, for either 12 or 18 weeks before exposure to oxygen. The condition of some animals, alive on decompression, deteriorated very rapidly within a few hours and these were killed. The term 'survival' is used only of those alive and well 24 hr after decompression. Because the rats were not all killed immediately on decompression, lung damage was not treated statistically. Eight litters, each of 4 males and 4 females, were allotted the numbers I–VIII at random, and combinations of treatment, sex, exposure time and time on the experimental diet were fully randomized and applied to the individuals of each litter in ascending order of weight. Since only 64 rats were available, a ½ replicate of a 2^7 factorial experiment was used, confounding some of the interactions to give 8 blocks of 8. Three animals died before exposure.

Experiment 2. This experiment was designed to investigate the responses of rats to exposure to four different O_2 pressures following four different treatments. Again, 64 rats were used, consisting of 8 litters, each of 4 males and 4 females. The animals were arranged, for sex, treatments and O_2 pressures, in two replicates of a factorial design, in which certain interactions were partially confounded between litters. The four treatments were α-tocopherol acetate, E, (20 mg/week); methylene blue, M, (0·15 g/100 g diet); glutathione, G, (2 mg/g body wt.); no supplement, O, to the animals acting as controls. The animals were exposed at the age of 125–145 days, a period of 10 days unfortunately elapsing between exposure of the rats in the 75 Lb./sq.in. group which were dealt with first, and the remainder.

RESULTS

Experiment 1

General effects of diet. In none of the females exposed after 12 weeks on treatment was there any uterine pigmentation and the males had suffered no loss in weight of testicular tissue. Of the animals exposed after 18 weeks, on the other hand, all the females receiving no supplement (T_1), single dose of α-tocopherol (T_4), and glutathione (T_7), had obvious uterine pigmentation and all the males had an equally obvious degree of testicular atrophy. Methylene blue (T_8) completely prevented both these manifestations of vitamin E deficiency.

Dialuric acid haemolysis test. The dialuric acid haemolysis test has been widely used as a criterion of vitamin E deficiency. In terms of this test, the animals receiving no tocopherol (groups T_1, T_7 and T_8) were deficient in vitamin E, while those given tocopherol were not. An exception is group T_6 which received the smaller dose of γ-tocopherol, and which was fairly near the dividing line, if such a term be permissible, between deficiency and the reverse. The specimens in group T_7 were taken before injection of glutathione.

TABLE 1. Haemolysis in blood obtained from vitamin E-deficient rats exposed to O_2 under a pressure of 75 Lb./sq.in. for various periods of time. The effects of addition of tocopherols and of methylene blue to the diet and of a single injection of glutathione (Expt. 1)

			α-Tocopherol acetate			γ-Tocopherol			
Exposure time (min)	Sex	No addition T_1	20 mg/week T_2	1 mg/week T_3	25 mg, one dose T_4	20 mg/week T_5	1 mg/week T_6	Glutathione, 2 mg/g body wt. T_7	Methylene blue 0·15 g/100 g diet T_8
15	M.	36·6	0	0	0	0	0	0	*
15	F.	0·5	0	0	0	0	0	0	0·5
30	M.	11·3	0	0	7·5	0	0·1	0·1	80·0
30	F.	32·2	0	0	0	0	0	1·1	46·0
45	M.	55·7	0	0	*	0	18·3	37·9	53·6
45	F.	3·0	0	0	0	0	0	0	*
60	M.	80·0	0	6·7	2·4	0	0	12·1	80·0
60	F.	28·2	0	0	0	0	0	2·4	73·0

* Indicates 'died before exposure'.

Haemolysis. Large numbers of negative results, together with very variable values, made any formal analysis of variance invalid, but from consideration of the data given in Table 1 it can be seen that haemolysis was entirely absent, or very slight, in rats given tocopherol; in those given methylene blue or no addition, haemolysis was almost always severe, while the rats given glutathione were between these two extremes. It seemed likely that there was a real difference between group T_7 (glutathione) on the one hand and groups T_8 (methylene blue) and T_1 (control) on the other hand, so that some degree of protection against haemolysis *in vivo* was given by glutathione in the dose administered, although less than that given by the tocopherols. Length of time on diet and time of exposure had no effect on the severity of haemolysis. Haemolysis was rather more obvious in male rats than in females. No haemolysis was observed in any sample taken before exposure of the living animal, and subsequently exposed *in vitro*.

Death or survival. These results are given in Table 2. They were analysed using −1 for a death and +1 for a survival, the missing values being estimated. Survival rates were significantly higher in rats maintained on α-tocopherol acetate or the high dose of γ-tocopherol than in those given no tocopherol ($P<0{\cdot}01$). In the groups given the single dose of α-tocopherol acetate or the lower dose of γ-tocopherol the rates lay somewhere between these two sets. The death rate increased with the length of time of exposure to oxygen under pressure ($P<0{\cdot}01$). Male rats died significantly more often than did female rats ($P<0{\cdot}01$).

TABLE 2. Survival of vitamin E-deficient rats exposed to O_2 under a pressure of 75 Lb./sq.in. for various periods of time. The effects of addition of tocopherols and of methylene blue to the diet, and of a single injection of glutathione (Expt. 1)

			α-Tocopherol acetate			γ-Tocopherol		Gluta-thione	Methylene blue,
Exposure time (min)	Sex	No addition T_1	20 mg/ week, T_2	1 mg/ week, T_3	25 mg, one dose T_4	20 mg, week, T_5	1 mg/ week, T_6	2 mg/g body wt. T_7	0·15 g/ 100 g diet, T_8
15	M.	D	S	S	S	S	D	D	*
15	F.	D	S	S	S	S	S	S	S
30	M.	D	S	S	D	S	D	D	D
30	F.	D	S	S	S	S	S	D	D
45	M.	D	D	S	*	S	D	D	D
45	F.	D	S	S	D	S	D	D	*
60	M.	D	D	D	D	D	D	D	D
60	F.	D	D	S	D	D	D	D	D

* Indicates ‘died before exposure’. S = survived. D = died.

Experiment 2

General effects of diet. At autopsy all females maintained on the deficient diet only, i.e. in groups (G) and (O), showed the typical uterine pigmentation and the males testicular atrophy of the same degree as already described. Tocopherol and methylene blue in the doses given again completely prevented those manifestations of vitamin E deficiency. The dialuric acid haemolysis test was positive in all animals not given tocopherol.

Nervous system disturbances. Mean values for time to onset of severe poisoning were used to construct the graphs shown in Fig. 1. At the lower pressures—below, roughly, 80 Lb./sq.in.—tocopherol gave a very high degree of protection, but at the higher pressures this protective power was lost. Both methylene blue and glutathione gave a much smaller degree of protection at these lower pressures ($P<0{\cdot}05$). Any such protection by methylene blue disappeared at higher pressures, but that given by glutathione appeared, on statistical analysis, to be retained even at the highest pressure employed. In fact, at an O_2 pressure of 115 Lb./sq.in. all rats given tocopherol and all given methylene blue became convulsed, and since this was the first evidence of toxicity, determination of time to onset of severe poisoning was exact. Of the eight control and glutathione-treated animals exposed to this pressure, only one became convulsed, and determination of the end point with equal accuracy was impossible. There was no significant difference between male and female rats.

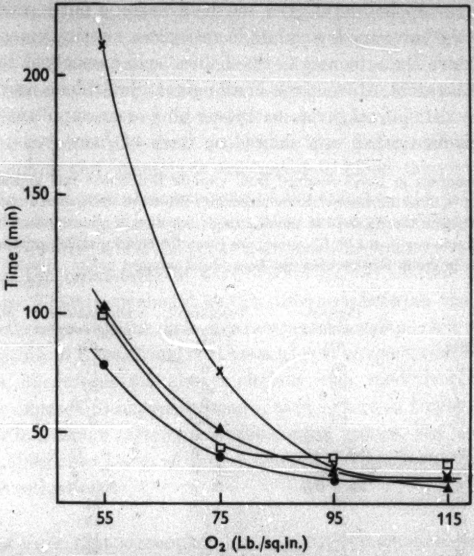

Fig. 1. Time to onset of severe poisoning in vitamin E-deficient rats exposed to different pressures of O_2. Effects of addition of α-tocopherol acetate and of methylene blue to the diet, and of a single injection of glutathione. Each point on the graphs represents the mean of 4 observations. ×, α-tocopherol acetate (20 mg/week); ▲, methylene blue (0·15 g/100g diet); □, glutathione (2 mg/g body wt.); ●, control (no addition).

TABLE 3. Lung damage in vitamin E-deficient rats exposed to O_2 under different pressures until the onset of severe poisoning. Effects of addition of α-tocopherol acetate and of methylene blue to the diet, and of a single injection of glutathione. Each value is the mean of 4 observations (Expt. 2)

Pressure Lb./sq.in.	Tocopherol (T)	Glutathione (G)	Methylene blue (M)	Control (O)
55	0	0	3·25	2·5
75	0	0·25	1·75	0·5
95	3·4	0	2·5	2·75
115	4	0	4	3·0

Lung damage. Tocopherol was highly efficient in protecting against lung damage at the lower pressures, but failed to give protection at high pressures. Methylene blue had no effect. In glutathione-treated animals, however, lung damage was negligible at all pressures. Mean values are given in Table 3.

Death or survival. These results are given in Table 2. They were analysed using −1 for a death and +1 for a survival, the missing values being estimated. Survival rates were significantly higher in rats maintained on α-tocopherol acetate or the high dose of γ-tocopherol than in those given no tocopherol ($P < 0{\cdot}01$). In the groups given the single dose of α-tocopherol acetate or the lower dose of γ-tocopherol the rates lay somewhere between these two sets. The death rate increased with the length of time of exposure to oxygen under pressure ($P < 0{\cdot}01$). Male rats died significantly more often than did female rats ($P < 0{\cdot}01$).

TABLE 2. Survival of vitamin E-deficient rats exposed to O_2 under a pressure of 75 Lb./sq.in. for various periods of time. The effects of addition of tocopherols and of methylene blue to the diet, and of a single injection of glutathione (Expt. 1)

			α-Tocopherol acetate			γ-Tocopherol			
Exposure time (min)	Sex	No addition T_1	20 mg/ week, T_2	1 mg/ week, T_3	25 mg, one dose T_4	20 mg, week, T_5	1 mg/ week, T_6	Gluta-thione 2 mg/g body wt. T_7	Methylene blue, 0·15 g/ 100 g diet, T_8
15	M.	D	S	S	S	S	D	D	*
15	F.	D	S	S	S	S	S	S	S
30	M.	D	S	S	D	S	D	D	D
30	F.	D	S	S	S	S	S	D	D
45	M.	D	D	S	*	S	D	D	D
45	F.	D	S	S	D	S	D	D	*
60	M.	D	D	D	D	D	D	D	D
60	F.	D	D	S	D	D	D	D	D

* Indicates 'died before exposure'. S = survived. D = died.

Experiment 2

General effects of diet. At autopsy all females maintained on the deficient diet only, i.e. in groups (G) and (O), showed the typical uterine pigmentation and the males testicular atrophy of the same degree as already described. Tocopherol and methylene blue in the doses given again completely prevented those manifestations of vitamin E deficiency. The dialuric acid haemolysis test was positive in all animals not given tocopherol.

Nervous system disturbances. Mean values for time to onset of severe poisoning were used to construct the graphs shown in Fig. 1. At the lower pressures—below, roughly, 80 Lb./sq.in.—tocopherol gave a very high degree of protection, but at the higher pressures this protective power was lost. Both methylene blue and glutathione gave a much smaller degree of protection at these lower pressures ($P < 0{\cdot}05$). Any such protection by methylene blue disappeared at higher pressures, but that given by glutathione appeared, on statistical analysis, to be retained even at the highest pressure employed. In fact, at an O_2 pressure of 115 Lb./sq.in. all rats given tocopherol and all given methylene blue became convulsed, and since this was the first evidence of toxicity, determination of time to onset of severe poisoning was exact. Of the eight control and glutathione-treated animals exposed to this pressure, only one became convulsed, and determination of the end point with equal accuracy was impossible. There was no significant difference between male and female rats.

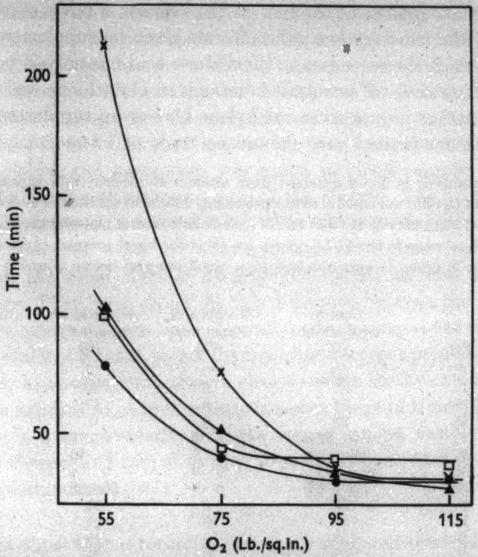

Fig. 1. Time to onset of severe poisoning in vitamin E-deficient rats exposed to different pressures of O_2. Effects of addition of α-tocopherol acetate and of methylene blue to the diet, and of a single injection of glutathione. Each point on the graphs represents the mean of 4 observations. ×, α-tocopherol acetate (20 mg/week); ▲, methylene blue (0·15 g/100g diet); □, glutathione (2 mg/g body wt.); ●, control (no addition).

TABLE 3. Lung damage in vitamin E-deficient rats exposed to O_2 under different pressures until the onset of severe poisoning. Effects of addition of α-tocopherol acetate and of methylene blue to the diet, and of a single injection of glutathione. Each value is the mean of 4 observations (Expt. 2)

Pressure Lb./sq.in.	Tocopherol (T)	Glutathione (G)	Methylene blue (M)	Control (O)
55	0	0	3·25	2·5
75	0	0·25	1·75	0·5
95	3·4	0	2·5	2·75
115	4	0	4	3·0

Lung damage. Tocopherol was highly efficient in protecting against lung damage at the lower pressures, but failed to give protection at high pressures. Methylene blue had no effect. In glutathione-treated animals, however, lung damage was negligible at all pressures. Mean values are given in Table 3.

Haemolysis. As in Expt. 1, formal statistical analysis was impossible. From inspection of the figures in Table 4, it is quite clear that tocopherol gave complete protection at all pressures investigated. The rats given methylene blue or no supplement showed, on the average, a fairly severe degree of haemolysis; the two very low values in rats given no supplement were from animals in which the responses to the dialuric acid haemolysis test were just positive and no more. Haemolysis in rats given glutathione was on the average less than that in rats given methylene blue or no supplement. Further, three glutathione-treated rats showed no trace of haemolysis; a complete absence of haemolysis, even after the specimen had stood for a few hours, was not demonstrated in any control or methylene blue-treated rat in Expts. 1 and 2. If the results of these 2 experiments are combined then there are 6 zero values out of 24 in the glutathione group and none out of 24 in the control group. This difference is significant ($P < 0{\cdot}05$). It seems legitimate therefore to assume that some degree of protection was given by glutathione. There appeared to be no correlation between the degree of haemolysis and the pressure of oxygen.

TABLE 4. Haemolysis in blood obtained from vitamin E-deficient rats exposed to different pressures of O_2 until the onset of severe poisoning. Effects of addition of α-tocopherol acetate and of methylene blue to the diet and of a single injection of glutathione. The values in the right-hand columns in the 75 Lb. group are those for blood samples exposed *in vitro*. The four values in group G were obtained from blood sampled before injection of glutathione (Expt. 2)

Pressure (Lb./sq.in.)	Sex	Control no addition (O)	Tocopherol, 20 mg/week (T)	Glutathione, 2 mg/g body wt (G)	Methylene blue 0·15 g/100 g diet (M)
55	F.	50	0	17	37
55	F.	60	0	20	68
55	M.	64	0	17·5	69
55	M.	85	0	6	72
75	F.	0·5 21·5	0 0	0 5	32 26
75	F.	0·5 0·5	0 0	0 13	30 50
75	M.	40 95	0 0	0 4	60 8
75	M.	7·5 0·5	0 0	36 9	50 60
95	F.	18	0	31	52
95	F.	25	0	25	17
95	M.	25	0	18	59
95	M.	52	0	25	80
115	F.	25	0	5·5	80
115	F.	63	0	6·5	68
115	M.	97	0	1·0	60
115	M.	95	0	1·0	91

In the blood samples taken before exposure of the animals, and exposed *in vitro* along with the animals to an oxygen pressure of 75 Lb./sq.in., no haemolysis was demonstrated where tocopherol had been given, but it occurred in all the other samples. Glutathione had not yet been injected when the blood samples were taken.

When the tubes, which had been centrifuged and stood in normal air, were examined after 24 hr, it was found that in every tube containing blood from a rat not given tocopherol there was a layer of richly haemoglobin-stained fluid, 1–3 mm in depth, lying immediately above the packed cells. There was no trace of haemolysis in blood from any tocopherol-treated rat.

There remained at the conclusion of this experiment a litter of seven rats. Three of these had been reared on exactly the same basic diet as the control experimental rats, and four of them had been given the diet with methylene blue. These rats were exposed to oxygen at atmospheric pressure. The three reared on the deficient diet died in 6–18 hr; they had passed urine stained with haemoglobin, and haemolysis was noted in blood samples taken and treated in the usual way after exposure of the animals. The four rats given methylene blue were alive and well after 24 hr and none showed any trace of haemolysis. However, when the tubes were stood in ordinary room air for a further 12 hr, the usual layer of haemoglobin-stained solution was seen in every instance. That there should be this difference between the two groups in the time taken for obvious signs of poisoning to develop is not surprising in view of the results of Expt. 2, and of the much lower oxygen pressure employed. Nevertheless, haemolysis was always obvious when methylene blue-treated animals were exposed to oxygen pressures above 1 atm in Expts. 1 and 2 and did not differ in average severity from the control groups. For these reasons, its complete absence at 1 atm of oxygen seems to be significant and deserving of further investigation.

DISCUSSION

These results show that α-tocopherol in any but the smallest doses gives a very considerable degree of protection against the effects of oxygen on the central nervous system and on the lungs, when compared with the effects of oxygen in vitamin E deficiency. This confirms the work reported in the earlier paper (Taylor, 1956). Even small doses of tocopherol give almost complete protection against haemolysis.

The results also seem to show that these three manifestations of oxygen poisoning arise independently of each other. Thus methylene blue gives some protection against the nervous system damage, but none against lung damage or haemolysis; and the protection given by tocopherol against haemolysis is very much greater than against nervous system or lung damage. Further, in Expt. 2, nervous system and lung damage increased with increase in O_2 pressure, but haemolysis did not. The results in Expt. 2 also suggested that tocopherol prevented lung damage at lower but not at higher pressures, while glutathione appeared to be effective over the whole pressure range. However, in Expt. 1, which was conducted within the lower range of pressures, lung damage did occur in both tocopherol- and glutathione-treated rats.

These results seem to give further support to the hypothesis of Bean & Bohr (1944) that oxygen under pressure has a damaging effect on essential enzymes, so that an actual tissue anoxia may result, and to the suggestion of Dickens (1946) that it is SH-containing enzyme systems which are involved. The results in the earlier paper (Taylor, 1956) did not show an unequivocal difference in time to onset of severe poisoning between tocopherol- and methylene blue-treated rats. That there is such a difference is now abundantly clear (Fig. 1). This difference may be explained by assuming with Hickman & Harris (1946) that tocopherol acts both to reduce the rate of cell metabolism and also as a general antioxidant. It would thus give protection from two directions, so to speak. Methylene blue and glutathione on the other hand, which might prevent the destruction of SH-containing enzymes, have been shown to increase oxygen consumption (Booker, Tureman, Da-Costa, Poulton & Mitchell, 1954; Bruns & Rummel, 1950). It is probably wise not to lay too much stress on the protection apparently given by glutathione at very high oxygen pressures, in view of the difficulty in determining precisely the time to onset of severe poisoning. The protection given by methylene blue and by glutathione in Expt. 2 was not reflected in the 24 hr survival rates in Expt. 1, survival in these groups being as poor as in the controls. Only with α-tocopherol in doses of 20 mg and 1 mg/week, and with γ-tocopherol in the higher dose, were survival rates high 24 hr after exposure. Admittedly the degree of haemolysis in five out of six animals given methylene blue in Expt. 1 was such as almost to preclude survival. Sixty minutes is greatly and 45 min just in excess of the average time to onset of severe poisoning recorded for those glutathione-treated animals in Expt. 2 which were exposed to oxygen at 75 Lb./sq.in.; none the less one glutathione-treated animal, exposed for only 15 min, and with no trace of haemolysis, was grossly ataxic and anorexic within 18 hr of exposure and had to be killed, and of the two animals exposed for 30 min one was killed on the day of exposure and the other was dead on the morning following its exposure.

The precise cause of the haemolysis in vitamin E-deficient rats exposed to oxygen is not at all clear. The conventional red cell fragility test, carried out on blood from a number of surplus deficient and methylene blue-treated rats gave no evidence at all of an increase in cell fragility. Christensen, Gortner & Dam (1955) observed haemolysis of the red cells of vitamin E-deficient rats, when the cells were suspended in 0·9% NaCl solution and incubated at 37° C under ordinary atmospheric conditions for 4 hr. If the incubation temperature was raised to 47° C haemolysis occurred in 1 hr. Räihä (1954) exposed samples of normal adult human blood to pure oxygen at atmospheric pressure, and at different temperatures, for periods of 1–24 hr, and also in various oxygen concentrations at 37° C for 24 hr. He stated that in some cases haemolysis occurred, but he did not give the number of positive and negative results. Vitamin E given in doses of 100 mg, 24 hr before the blood samples were taken, inhibited haemolysis. The observation in Expt. 2 that vitamin E-deficient red cells were haemolysed fairly quickly when exposed *in vitro* to oxygen at high pressure and at a temperature, initially, of 40° C, fits in with these observations. So, too, does the finding that such cells become haemolysed in ordinary air at room temperature within 24 hr. Haemolysis occurred *in vitro* during exposure to oxygen only when complete saturation of the samples with oxygen was ensured by stirring. It is impossible on the available evidence to say whether haemolysis *in vivo* is due to a similar direct action of oxygen at high pressure on the red cells, or whether in the intact animal some metabolic process intervenes, or fails to intervene. Certainly the discrepancies between the values for haemolysis *in vivo* and *in vitro* might have been due to the rather crude methods employed in dealing with the *in vitro* samples such as rapid stirring and lack of temperature stabilization. No haemolysis was ever found in blood examined soon after withdrawal from an animal exposed only to ordinary atmospheric conditions; and for the four methylene blue-treated rats at the conclusion of Expt. 2, no haemolysis was seen immediately after exposure to oxygen at 1 atm, although haemolysis occurred when the samples were left during the night in the laboratory. There seems to be no obvious explanation, also, for the lack of correlation between haemolysis and either length of exposure or oxygen pressure. One might postulate different rates of absorption of the subcutaneously injected solution in the case of glutathione, but the range of values was just as wide in the control and methylene blue-treated groups. The fact remains that vitamin E-deficient red cells are liable to undergo haemolysis under a wide variety of experimental circumstances, and that administration of even small amounts of tocopherol for more than the briefest period gives protection. In this respect α-tocopherol seems to be rather more efficient than γ-tocopherol. Gerschman and her colleagues in a number of recent papers (Gerschman, Gilbert, Nye, Dwyer & Fenn, 1954; Gerschman, Gilbert, Nye & Fenn, 1954; Gerschman, Nye, Gilbert, Dwyer & Fenn, 1954; Gilbert, Gerschman & Fenn, 1956) have drawn an analogy between oxygen poisoning and radiation poisoning, postulating the formation, as a result of exposure to oxygen under high pressure, of the free radicals H^+ and OH^- with subsequent formation of H_2O_2 and HO_2^- which are believed to be formed as a result of X-irradiation. They found, too, that a number of substances such as cysteine, β-mercaptoethylamine, reduced glutathione and tocopherol, protected against either type of poisoning.

These observations are clearly of importance when one attempts to elucidate the cause or causes of oxygen poisoning in general, quite apart from the haemolysing action on red cells in vitamin E deficiency. In fact, in this respect, the protection given by tocopherol is so great as to suggest that it has an independent direct effect on the red cells, possibly on the structure of the membrane. In the absence of more evidence, however, this must remain mere speculation.

SUMMARY

1. The effects of vitamin E deficiency and of treatment with tocopherols, methylene blue and glutathione were investigated in rats exposed to oxygen for different periods of time and under different pressures.

2. Vitamin E-deficient rats showed a decreased resistance to the effects of oxygen on the central nervous system, when compared with rats treated with α-tocopherol acetate. Methylene blue and glutathione had a smaller but definite protective effect.

3. Lung damage was less in rats given α-tocopherol acetate or glutathione than in those given methylene blue or no supplement.

4. Survival rates were high in rats maintained on α-tocopherol acetate and large doses of γ-tocopherol, intermediate in those given small doses of γ-tocopherol or one large dose of α-tocopherol acetate, and poor in animals given glutathione, methylene blue, or no supplement.

5. All vitamin E-deficient, untreated rats exhibited haemolysis *in vivo* following exposure to abnormally high oxygen pressures. Tocopherol, in any but the smallest doses, gave complete protection; γ-tocopherol was less efficient than α-tocopherol acetate. Glutathione gave some protection. Methylene blue gave no protection, except in four animals exposed to pure oxygen at atmospheric pressure. Haemolysis was greater in male than in female rats.

6. Red cells from vitamin E-deficient rats underwent haemolysis *in vitro* when exposed to oxygen at a pressure of 75 Lb./sq.in. for less than 1 hr, and also in ordinary room air within 24 hr. This haemolysis was absolutely prevented by α-tocopherol acetate (20 mg/wk) and was not affected by methylene blue.

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DATA FOR THE CALCULATION OF THE RATE COEFFICIENTS OF SODIUM TRANSFER BY MOUSE ASCITES TUMOUR CELLS

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Cation transport in mammalian erythrocytes is energized by glycolysis (Harris, 1941; Maizels, 1951); in chicken and tortoise erythrocytes glycolysis is normally slight, and transport is based on respiration (Clarkson & Maizels, 1955; Maizels, 1956). In the case of tumour cells respiration is active, but glycolysis proceeds simultaneously even in the presence of oxygen (for references see Warburg, 1956); it therefore seemed of interest to examine the contribution of these activities to cation transport by tumour cells. The cells of the ascites tumour (Ehrlich) of mice were selected for study, because, like erythrocytes, they are suspended in a fluid medium and can be centrifuged; hence they may be examined by methods appropriate to the study of erythrocytes.

The present paper is concerned with the estimation of Na, K and water in ascites cells, with the measurement of the amount of intercellular fluid in centrifuged cells, and with other matters relevant to the calculation of the rate coefficients for Na transfer by the cells.

METHODS

Transfer coefficients were measured by the 'chemical method' of Harris & Maizels (1951). In practice this involves raising the Na concentration of ascites cells by suspension in potassium-free media at 4° C, then warming, initiating Na transfer by adding potassium, and measuring the fall in Na concentration with time.

Ascites tumour fluid from mice (C- strain) was collected in heparin solution (2 mg/100 ml.). If blood-stained (and this was usual) it was centrifuged, the supernatant removed, and the cells resuspended in NaCl solution (0·16 M) and centrifuged slowly (about 60 *g*); the ascites cells (presumably because of their high ratio of volume to surface area) were centrifuged down, while the erythrocytes remained suspended (washed cell preparations). In some cases further preliminary treatment was needed: thus when investigating the action of metabolic poisons it was necessary to deplete the cells of some of their natural reserves of energy. This was achieved by washing, incubating at 38° C for an hour and cooling (depleted cell preparations). However prepared, the cells were ultimately transferred to a medium containing 1·75 mM-$CaCl_2$, 1 mM-$MgCl_2$, 127·5 mM-NaCl, 12·5 mM-Na succinate and 25 mM glucose, as well as 3 g human serum albumin and 1·75 mg phenol red/100 ml. At the start of observations enough KCl was added to give 8 m-mole/l.

medium. The actual Na content varied between 140 and 165 m-equiv/l. owing to variable amounts of Na in the albumin added; sometimes one or other constituent was omitted, or metabolic poisons or other agents might be added. The cells contributed 2% by volume.

Before observations were begun, part of the contents of each flask was transferred to a Warburg flask, part was removed for analysis, and the rest gently rocked at 35–38° C, the flasks being closed with rubber bungs, which were removed at intervals for further sampling. Such suspensions rapidly became very acid (pH 6·5 or less) and therefore 0·15 M-NaOH in 0·008 M-KCl was added at intervals to keep the pH about 7·3. The buffering effect of protein present helps to prevent shift of pH, but the addition of 2-amino-2-hydroxymethylpropane-1:3-diol (tris) to 50 mM was virtually useless, and this buffer was discarded. In the case of cell suspensions in Warburg flasks, the correct pH was maintained by tipping in small amounts of dilute NaOH from the side arm, from time to time.

It will be noted that a fairly dilute cell suspension was used, not only to economize in material, but also to minimize the necessity for too frequent additions of alkali, a matter of importance when lactic acid may exceed 150 μmole/ml. cells. hr. With a 2% suspension of cells, oxygen consumption could be measured satisfactorily on 3 ml., while lactate estimations (Hullin & Noble, 1953) required 0·25 ml. Since the original ascites fluid contained 20–40% of cells a 10–20-fold dilution was used. When the diluent contained only crystalloid, the cells of the suspension rocked at 35–38° C rapidly aggregated to form large flakes. This could be prevented by adding human serum albumin (to 3%, w/v), and although cell-free ascites fluid would have provided an ideal diluent, albumin was preferred in order to avoid the unnecessary slaughter of mice.

Na and K in ascites cells were estimated in the same way as in erythrocytes. A number of special tubes were prepared, whose upper halves were wide (about 12 mm in diameter), while the lower halves were drawn out to sealed graduated and calibrated capillaries. The tubes were placed in a small water-bath at the appropriate temperature, and measured volumes of suspension from each flask added to paired tubes, estimations being done in duplicate. When all were ready, the tubes were rapidly transferred from the bath to the centrifuge buckets, and centrifuged at 2000 *g* for 25 min, after which the volume of the packed cells (about 0·03 ml.) was carefully read with a hand lens. The upper part of the tubes above the packed cell column was next well washed, and the cells suspended in known amounts of water (100–200 vol.). After 15 min the water suspension was well shaken, the open end of the tube being closed with the thumb covered with a clean rubber finger-stall. Cellular debris was centrifuged down, and Na and K in the supernatant estimated with an EEL flame photometer, the difference between duplicates seldom exceeding ±1%. The figures agreed well with parallel results obtained by ashing unlysed cells and extracting with HCl. The following are examples: Na—102·5 and 100·4 compared with 99·3 and 98·7; 30·0 and 29·3 compared with 30·3 and 29·8; K—50·0 and 48·6 compared with 50·7 and 49·9; 20·1 and 19·7 compared with 20·0 and 20·0 m-equiv/l. cells.

Chloride was estimated by the method of Claudius (1924); error ±1·5%. When radioactive tracers were used a measured amount was counted in a liquid counter.

At the end of an experiment, the pH of each of the various supernatants from the centrifuged cell suspensions was measured at 20° C with a glass electrode.

PART 1. SOME PROPERTIES OF THE ASCITES TUMOUR CELLS OF MICE

In dealing with ascites cells it was necessary to establish certain data of a general nature, and it is important to realize that tumour cells are much more variable than are normal tissues.

Effects of time of centrifuging on the volume of the centrifugate. It was thought that 25 min sufficed to give consistent packing at 2000 *g*. The following is an example: after ½ min centrifuging the volume of the cell mass was 0·080 ml., at 5 min 0·061, at 10 min 0·056, at 15 min 0·054 and at 30 min 0·052 ml.

Volume of the intercellular space. Evan's blue which is appropriate for measuring the intercellular space of erythrocytes (Maizels, 1945) is unsuitable here, because it penetrates into, or is adsorbed on the ascites cells. The protein of the trapped ascites fluid was also tested as a marker, by resuspending the centrifuged cells in NaCl solution, recentrifuging, and comparing the protein washed out in the supernatant saline with that of a standard dilution of the original ascites fluid in which the cells were suspended. This method gave values of about 30 % for trapped fluid, and it was thought that additional protein had been contributed by cytolysis in the NaCl washing fluid, and had thus invalidated the method. As an alternative, a haemoglobin solution (6 g/100 ml. NaCl solution 0·9 %) was added as marker: this gave values for ten different cell batches of 13·9 % (S.D. ± 1 %). In three other experiments, with iodinated albumin (labelled with ^{131}I) as marker, the figures were 13·5, 15·3 and 14·9, but with stored iodinated albumin higher figures were obtained—17·3 and 17·8 %: possibly surface-active iodine compounds had formed on keeping. If then it be assumed that neither haemoglobin solution nor fresh iodinated albumin is adsorbed on ascites cells, it follows that trapped fluid contributes about 14 % to the packed cell volume, and observed cell data should be corrected for this amount of trapped medium to obtain net values.

Percentage cell volume. With C- mice freshly inoculated from a C 57 strain, ascites cells constitute 30–35 % of the fluid, but after repeated passage through C- strains the percentage may fall to 20 or less. It may be noted that in most cases the ascites fluid was haemorrhagic.

Composition of ascites fluid. The pH of the fresh fluid was about 7·3 (7·28, 7·30 and 7·36) measured at 20° C, but it rapidly became more acid unless separated from the cells. Protein in the cell-free fluid was very variable: 3·7 g/100 ml. in one sample and only 1·4 in a second; presumably the value depends on the degree of peritoneal reaction and on the amount of cytolysis. Potassium was also variable, being 7·3, 8·8, 11·4, 11·6 and 11·8 m-equiv/l. in five fluids, the figure depending in part perhaps on the amount of cytolysis, and also on haemolysis in haemorrhagic fluids. Delay in separating the cells also increased extracellular K, especially at low temperatures. The Na contents of five samples of cell-free fluid were 140, 145, 149 and 153 m-equiv/l.

Size of ascites cells. Here it was necessary to use non-haemorrhagic fluids; these were infrequent and only four samples were examined. Most of the cells were about the same size, with some small cells, possibly lymphocytes, and others of intermediate size. The gross mean cell volumes were 4000, 3400, 3000 and 2600 μ^3; the net volumes (volume of the intercellular space deducted) were 3400, 2900, 2600 and 2200 μ^3. If it be assumed that the cells are spherical, then the corresponding diameters are 18·5, 17·7, 17·0 and 16·2 μ, and the respective areas are 1080, 980, 910 and 820 μ^2. There is thus a good deal of variation in the dimensions of different samples, but when converting sodium fluxes, which are here given in m-equiv/l. cell water. hr to the alternative units pmole/cm^2. sec, it may be assumed that cell volume is roughly 2800 μ^3, area 940 μ^2, and cell water about 86 % (see below), in which case the conversion factor is 0·07.

Composition of fresh ascites cells. When examined within 5 min of aspiration, water content was fairly constant, averaging 88·2 % (w/v) in ten observations with a range of 87–90 %. Na and K were very variable, the former averaging 54 m-equiv/l. cells (range 40–65 m-equiv) and the latter averaging 98 m-equiv/l. cells (range 111–79 m-equiv). If these values are corrected for intercellular contributions, the mean net contents are: water 86 %, Na 39 and K 113 m-equiv/l. cells. Hence the average content of Na + K in the cells is slightly lower than that of the natural suspending fluid, but owing to the lower water content of the cells their concentration is about 11 % higher than that of the fluid. A similar excess of cell base concentration is found with cells incubated in artificial media (Maizels, Remington & Truscoe, 1958).

When the ascites cells have been processed as described in the section on Methods (washed, cooled and then transferred to the standard medium), their volume after ½ hr incubation at 35° C is about 10 % greater than that of the fresh cells and their water content about 1 % greater.

Chloride distribution in suspensions of ascites cells. The chloride distribution in fresh material was not determined, but for the purposes of subsequent experiments it was measured in suspensions of cells in the standard experimental medium (see Methods). With cells subjected to minimal treatment (washing only) the ratio of the external to the cell chloride concentration ($[Cl_e]:[Cl_i]$) was 129:61 or 2·1 at pH 7·3, and 129:60 or 2·2 at pH 7·4. For cells depleted of metabolic reserves by washing, preliminary incubation for 1 hr in glucose-free media, followed by cooling and further incubation for 15 min (a method used when investigating the action of metabolic poisons), the chloride concentration ratio was 128:79 or 1·62 at pH 7·29, and 128:74 or 1·73 at pH 7·51. The relatively low ratio resulting from prolonged incubation may be due to loss of indiffusible anion, possibly by dephosphorylation of phosphoric esters. Other chloride ratios for different batches of washed and depleted cells were 1·56 and 1·64, while in the case of a single batch of cells, the following ratios were obtained: at pH 7·23, 1·45; at pH 7·38, 1·55; at pH 7·38 in the presence of iodoacetate (0·05 mM), 1·42.

It may be noted that while Na in the artificial suspending medium is about the same as that of natural fluid (150 m-equiv/l.), the chloride in the former is possibly less, because while it lacks natural bicarbonate 12·5 mM-Na succinate has been added as substrate.

The metabolism of ascites cells. This is discussed more fully below, though it may be noted here that O_2 consumed ranges between 1000 and 2000 μl./ml. cells. hr, while lactate formed varied between 100 and 300 μmole/ml. cells. hr.

Effects of temperature on the cation content of ascites cells. When ascites cells are cooled to 4° C cations flow with the concentration gradients; on incubation at 35° C in the presence of K the flow is reversed, Na and K moving against the gradients. The effects of temperature on this active transport are discussed more fully in a subsequent paper.

Effects of washing ascites cells with NaCl or KCl solutions. In view of the large contribution of intercellular medium to the gross composition of centrifuged ascites cells, it would be advantageous if the medium could be removed by washing the cells in a suitable solution. In the case of animal erythrocytes permeation is sufficiently slow to permit the removal of intercellular fluid by rapidly washing with KCl or LiCl solution (Clarkson & Maizels, 1955), but with ascites cells a single rapid wash with KCl solution lowers cell Na by 30–40 m-equiv/l. Thus, in one experiment where centrifuging was delayed for 5 min, the gross actual loss at 18° C was 51 m-equiv/l. cells, corresponding to a net loss of 30 m-equiv/l. after deducting Na removed in the intercellular fluid. In another experiment with cells washed in NaCl solution (instead of KCl) at 18° C, there was a rise in Na (after deduction of intercellular contributions) of 23 m-equiv/l. cells in 5 min. It follows that washing ascites cells to remove intercellular medium is inadmissible.

Effects of adding radiosodium (Na*) *to suspensions of ascites cells.* In the following, a calibration factor has been used converting counts per min to m-equiv. As might be expected from the preceding paragraph, entry of Na* into erythrocytes is extremely rapid. Even if the cells are centrifuged as soon as possible after the addition of tracer, the value for cell Na* may almost have reached that of cell Na. Thus, in an experiment at 35° C at '0' min gross cell Na (i.e. total Na in packed cells uncorrected for intercellular Na) was 51·7 m-equiv/l., and gross Na* was already 48·6 m-equiv/l. cells, corresponding to an entry of 27 m-equiv/l. of Na* into the cells proper. In another experiment at 25° C gross cell Na and Na* at '0' min were 118 and 68·5 m-equiv/l., the latter corresponding to an entry into the actual cells of 47 m-equiv Na*/l. It may be noted that within 15 min of the addition of Na* cell concentrations of Na and Na* were virtually equal, indicating that all the Na of ascites cells is exchangeable.

The 'centrifuge error'. It is clear that the very rapid exchanges of cation described above must occur during the essential manipulations and during the early part of centrifuging before the cells are fully packed, and it may be thought that this 'centrifuge error' constitutes a bar to measuring the cation content of cells not at equilibrium with the external medium. This is in fact the case when cations are freely exchangeable, but does not hold for actively transporting cells, where the exchange is restricted. It is necessary to consider this matter more fully, since the measurement of changes in the cation content of actively transporting cells is the basis for the method proposed of calculating transfer coefficients.

When the rate coefficients for Na efflux and influx (k_2 and k_1) are respectively about 10 and 2·5/hr, [Na_i] will fall in 5 min from 80 m-equiv/l. cell water after keeping at 4° C, to 40 m-equiv/l. at 35° C, so that the time taken to pack the cells by centrifuging the warm suspension might be highly significant; moreover, the error due to time of centrifuging is not constant, but becomes progressively less when the cells have approached equilibrium before centrifuging. However, the centrifuge error is not as great as the rate coefficients might suggest, for after only half a minute's centrifuging, when intercellular medium still contributes about 30 % to the settled cell volume (instead of the final 14 %), further Na exchange virtually ceases. This arises as follows: with cells in a small volume of medium, Na efflux can only occur in two ways; (*a*) by exchange with external potassium, (*b*) by the continued excretion of Na not in exchange for K. With regard to the former, if one assumes that after centrifuging for 30 sec, 3 vol. of cells are now suspended in 1 vol. of medium containing 8 m-equiv K/l., then if all this K were removed in exchange for Na, Na lost by the cells would be less than 3 m-equiv/l. This view was confirmed as follows: cells loaded with Na at 4° C were warmed, centrifuged for 30 sec, the supernatant removed completely (first supernatant) and the deposit recentrifuged for a further 20 min. The volumes of the cells and residual supernatant were measured, and the latter (second supernatant) removed for analysis. In one experiment the first supernatant contained 7·9 m-equiv K and 156 m-equiv Na/l., while the second supernatant contained 3·4 m-equiv K and 160 m-equiv Na/l. The volume of the second supernatant plus the volume of trapped medium in the fully packed cells at 20 min was 36 % of the true net cell volume; hence gain of K by the cells between $\frac{1}{2}$ and 20 min was 1·6 m-equiv/l. cells while loss of Na was 1·4 m-equiv/l. In other experiments, loss of Na during centrifuging was 1·9, 0·9 and 1·4 m-equiv/l. cells. These changes are negligible when compared with the exchanges of cells freely suspended in a large volume of medium. Na might also be lost from the cells by the continued excretion of Na together with an anion during centrifuging, not in exchange with external K. This would presumably be accompanied by a loss of cell water, and the Na concentration of the cells should be little affected. In any case unlinked excretion of Na, if it occurs at all, is small, and could only occur in K-poor media; if, as was the case with the supernatants described above, the external K concentration exceeds 2 m-equiv/l. active exchanges of Na and K probably proceed on a 1:1 basis (see below).

It will be realized, then, that cell sodium concentration changes little once centrifuging has begun, because after the first half minute there is little K in the extracellular 'shell' with which to exchange. If, however, the external medium is rich in K or Li, as in KCl or LiCl washes, cation exchange during centrifuging may be significant; so too, as already noted, Na* in the external medium can exchange freely with cell Na, and the specific activity continues to rise during centrifuging. Ordinarily, however, when estimating fall of Na by the simple 'chemical method' to be described presently, the 'centrifuge error' is quite insignificant, provided the speed is stepped up to its maximum at a constant and very high rate.

PART 2. MEASUREMENT OF THE RATE COEFFICIENTS FOR SODIUM TRANSFER

Use of tracers

In order to resolve a net change in cell Na into its components of influx and efflux, it may be supposed that a tracer technique would be appropriate, but in the case of ascites cells the difficulties are formidable. Thus trapped intercellular plasma constitutes about 14 % of the packed cell mass, and contributes about 22 m-equiv Na or Na*/l. packed cells or 30 m-equiv/l. cell water; such a zero value adds greatly to the over-all experimental error, which cannot be mitigated by washing the cells, since as has already been seen washing is inadmissible. Further, the figures suggest that, on the addition of Na*, the earliest possible observations will already show values for the amount of Na* in the packed cells far in excess of that trapped in the intracellular medium; such a high base line will interfere seriously with accuracy. Experience has confirmed this: thus in one experiment where Na* was added to a cell suspension ('soaking-in' method), Na and Na* at '0' min were respectively 106 and 85 m-equiv/l. cell water, *both* declining together to 85 and 75 m-equiv at 4 min: allowing experimental errors for Na of ± 1 % and for Na* of ± 1·25 %, and calculating the rate coefficient for Na efflux according to E. J. Harris (see Clarkson & Maizels, 1955) gives a value of 21 ± 9/hr.; in two other experiments, carried out at 25° C instead of 35° C in order to slow the transfer, the rate coefficient for efflux was calculated at 4·0 ± 1 and 10·7 ± 1·9. Clearly the tracer method as ordinarily used is inapplicable in view of the excessive uncertainty of the results.

Chemical method for measuring transfer coefficients

In view of the difficulties incurred by the use of tracers, the 'chemical' method for measuring transfer coefficients (Harris & Maizels, 1951) was used. This involves raising the cell Na concentration by suspending cells in K-free media at 4° C and following the fall of concentration with time on subsequent incubation with K. Then

$$d[\mathrm{Na_i}]/dt = \text{influx} - \text{efflux}, \qquad (1)$$

influx and efflux being expressed as m-equiv/l. cell water.hr. With the external phase very large, and assuming that influx is unaffected by the cell Na concentration, it follows that influx will be constant, and at infinite time, when $[\mathrm{Na_i}]$ has fallen to a constant low level, it will equal efflux; hence

$$d[\mathrm{Na_i}]/dt = (\text{Na efflux at time } \infty) - \text{Na efflux} \qquad (2)$$

If now it be assumed that Na efflux is a first-order process with a rate constant k_2, so that it is equal to $k_2[\mathrm{Na}_i]$, then equation (2) may be integrated:

$$k_2 t = \ln\,([\mathrm{Na}_i]_\infty - [\mathrm{Na}_i]_0)/([\mathrm{Na}_i]_\infty - [\mathrm{Na}_i]_t), \tag{3}$$

where $[\mathrm{Na}_i]_0$, $[\mathrm{Na}_i]_t$ and $[\mathrm{Na}_i]_\infty$ are the respective Na concentrations at zero time and at times t and ∞. k_2 is in fact not always constant, but may tend to decrease with time, and has therefore been called the rate coefficient. The assumption that Na efflux is a first-order process gains support from the observation that k_2 is little affected by initial values of $[\mathrm{Na}_i]$ (see table 4 of Maizels *et al.* 1958). It is true that when the *external* Na concentration is halved (80 mM choline chloride being added in replacement), k_2 falls by 0–10%, while the ratio $[\mathrm{Na}_i]_\infty : [\mathrm{Na}_e]$ rises by 0–12%, though both of these should be constant for a first-order process of transfer. It may be held, however, that in view of the unphysiological nature of the substance added to maintain the external osmotic pressure, the slightness of the observed deviations (Table 5 of Maizels *et al.* 1958) lends support to the assumption of a first-order process, and at least suggests that equation (3) may provide a consistent approximation to the true value of k_2.

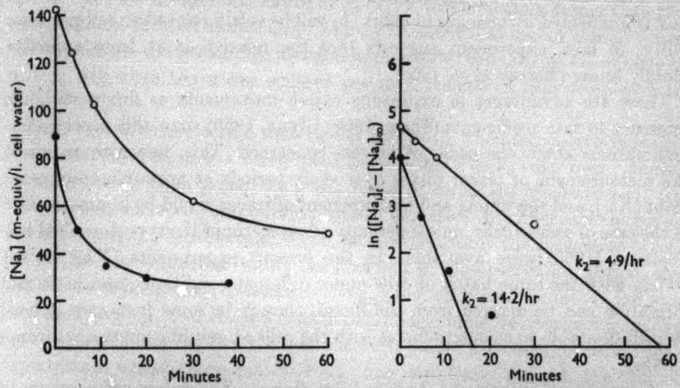

Fig. 1. Fall of cell Na concentration ($[\mathrm{Na}_i]$) on incubating Na-rich mouse ascites tumour cells at 35° C. Quickly transporting strain, ●. Slowly transporting strain, ○.

The assumption that Na efflux is a first-order process gains further support from the fact that the plot of $\ln\,([\mathrm{Na}_i]t - [\mathrm{Na}_i]_\infty)$ against time often approximates to a straight line, from whose slope a value for k_2 may then be obtained. However, in some experiments this curve is somewhat concave upwards (Fig. 1) suggesting that either the ascites cell itself, or the cell suspension, is not homogeneous. With regard to the first possibility, this must obviously exist in any nucleated cell, though data for radiosodium suggest that all cell Na is exchangeable: concerning the second possibility, it is a fact that in most experiments pooled material from two mice was used, and it is likely that differences in cell activity may have been a factor in deviations from linearity. When such deviations were more than slight, k_2 was found by graphic integration.

The rate coefficient for Na influx (k_1) was obtained (Harris & Maizels, 1951) from

$$k_1[\mathrm{Na_e}] = k_2[\mathrm{Na_i}]_\infty \tag{4}$$

This assumes that Na influx is also a first-order process. However, since in all experiments $[\mathrm{Na_e}]$, the external sodium concentration, is always about the same, the validity of the assumption is not important, and provided that influx is unaffected by changes in efflux, k_1 will be solely related to cell permeability; in fact, experiment suggests that the relation is at least approximately linear (Maizels *et al.* 1958).

There are advantages in expressing cation movements as fluxes without reference to rate coefficients (Shaw, 1955; Glynn, 1956) since this involves no assumptions about the order of process concerned. This, however, requires the measurement of tracer fluxes over short periods at approximately constant $[\mathrm{Na_i}]$, and the timing and measurement of tracer would be impracticable in the case of ascites cells, unless perhaps a flow-meter of the type described by Tosteson (1956) were available. In the present experiments $[\mathrm{Na_i}]$ varies widely with the same batch of cells under different conditions, and little information can be derived from the fluxes, though in some instances it has seemed desirable to calculate fluxes with the cells at equilibrium ($t = \infty$) from $k_2[\mathrm{Na_i}]_\infty$.

Changes in cell volume and the transfer coefficients. The volume of incubated cells is referred to a standard volume. The standard is taken from the control sample incubated for ½ hr in the presence of potassium and glucose and in the absence of metabolic poisons. In many experiments, when ascites cells have been charged with Na by keeping in the cold, a concentrated suspension is added to the warmed incubation medium containing K, and in these circumstances little change in volume occurs. But in experiments concerned with metabolism and transfer the cells must be equilibrated for 15 min in K-free medium at 35° C before K is added. Here the cells shrink in the warm K-free medium and return to about the normal volume after K is added. Correction for changes in volume is difficult because it is quite uncertain whether the corresponding changes in surface area will affect Na transfer, and even the effects of simple volume changes are obscure. The matter is discussed in a subsequent paper (Maizels *et al.* 1958), but in view of these difficulties, only those experiments have been used in which volume changes were less than 10%, or in which the volume changes though a little more than 10% were all similar for the differently treated samples contributing to an experiment. This was often the case even with poisoned suspensions, for the 'chemical method' requires that some active transport always remains, and in these circumstances volume changes in the partially poisoned suspensions and in controls were similar. In such cases k_2 has a relative rather than an absolute value.

Data for time curves. Having obtained gross values for cell Na after varying periods of incubation, it is necessary to obtain net values by deducting Na and water in the intercellular medium, which in cells packed at 2000 g is about 14 % by volume. The Na concentration of the cells ($[\mathrm{Na_i}]$) may then be calculated on the assumption that the standard cells under experimental conditions have a net water content of 87 % (w/v); this is slightly higher than the water content of fresh ascites cells. It may be noted that loss or gain of water will not change the percentage of water appreciably: thus changes in volume of ±10 % will alter the water content per unit volume of cells from 87·5 to 87·5 ± 1·2 %.

With regard to the actual time curves for $[\mathrm{Na_i}]$: when comparing four different suspensions, it was rarely possible to supply more than four 'points' for each curve (each point being the mean of duplicate observations); for three suspensions five-point curves could be prepared. In either case the curve was drawn with a flexible rule. When, as was commonly the case, most of the fall in $[\mathrm{Na_i}]$ is completed during the first half of the observation period, the last observed point may be taken as $[\mathrm{Na_i}]_\infty$ (Fig. 1). When the rate of fall was less rapid (e.g. fig. 3 of Maizels *et al.* 1958), $[\mathrm{Na_i}]_\infty$ must be obtained from the curve extrapolated beyond the last observed point. However, even in those experiments where the value of $[\mathrm{Na_i}]_\infty$ may be clearly defined, the plot of $\ln([\mathrm{Na_i}]_t - [\mathrm{Na_i}]_\infty)$ is not necessarily linear. Such plots fall into two groups: (*a*) The plot is nearly linear (Fig. 1), the last point alone being apparently too high. The deviation is of course magnified by the form of the equation on which it depends: thus the last point of Fig. 1 (lower curve) corresponds to an experimental value for $[\mathrm{Na_i}]_\infty$ of 28·5 m-equiv/l. cell water; a value of 29·9 m-equiv would in fact have made the whole curve approximately linear. In these circumstances it has seemed best to draw a line which ignores the last point of the curve (Fig. 1) and derive k_2 from this. In effect this involves assigning a slightly higher value to $[\mathrm{Na_i}]_\infty$ than is actually the case, and if from the data corresponding to Fig. 1 (lower curve) k_2 is calculated by equation (3) one obtains 13·0/hr using the observed value of $[\mathrm{Na_i}]_\infty$, and 13·8/hr with the assumed value; the corresponding values for k_1 are 2·3 and 2·4/hr. (*b*) The plot of $\ln([\mathrm{Na_i}]_t - [\mathrm{Na_i}]_\infty)$ deviates throughout from linearity: if the deviation is marked the experiment must be rejected, otherwise k_2 is obtained graphically from the plot of $[\mathrm{Na_i}]$ against time, the Na flux between $t = 0$ and $t = \infty$ being divided by the mean cell concentration. Data suitable for treatment by the first group (*a*) may also be used to provide a graphic solution by method (*b*). In forty consecutive observations k_2 obtained by the first method was on the average 1 % greater than when obtained by the second method, S.D. being ± 6 %. With a single cell suspension examined in triplicate, results for k_2 and also for k_1 were very close indeed, but when suspensions are not identical error may arise in the value assigned to $[Na_i]_\infty$. This is not likely to exceed ± 4 %, and with typical experimental data would give a maximum error in k_2 (obtained by any one method) of not more than ± 6 %. In view of the possible error in measuring k_2, it is doubtful whether importance should be attached to any apparent difference in k_2 values less than 15 %.

Rate coefficients in the presence and absence of phenol red

As a preliminary, it was desirable to show that phenol red which was always added to the standard medium as an indicator (see Methods) had no adverse effect on Na transport. To this end parallel suspensions were prepared, in only one of which phenol red was present. Equal amounts of NaOH were added as required, and the fall of $[Na_i]$ in the presence of potassium was observed. The respective figures for suspensions without and with phenol red were as follows: rate coefficient for Na efflux (k_2) 11·1 and 10·9/hr, and for Na influx (k_1) 5·3 and 5·1/hr; glucose converted to lactate 116 and 124 μmole, and glucose presumed converted to CO_2 11·2 and 11·9 μmole/ml. cells.hr. It follows that the dye had no adverse effect on cell activities.

The rate coefficient for Na efflux has varied widely between 3 and 18/hr for undepleted cells, and between 2 and 15/hr for depleted cells (cells depleted of metabolic reserves by preliminary incubation in glucose-free media), the average for the former (38 observations), being 9·1 and for the latter (69 observations) 7·8/hr. The rate coefficient for Na influx varied between 1 and 5 for undepleted cells (mean 2·2), and for depleted cells between 1 and 7·6 (mean 3·2), and although in parallel experiments with the same cells depletion sometimes seems to leave k_1 and k_2 unaffected, as a rule depletion affects Na transport adversely and also decreases the resistance of the cell to passive penetration. Typical curves for fall of $[Na_i]$ in quickly and slowly transporting cells are shown in Fig. 1.

DISCUSSION

Ascites cells are roughly spherical, and since about 14 % of the packed cells consist of trapped medium, it may be presumed that the cells undergo little deformation on centrifuging. The gross water content is about 88 % (w/v), which is greater than that of many solid organs, but closer to that of leucocytes, which according to Hempling (1954) contain 79 % water (w/v), corresponding to about 84 % (w/v).

The ratio of external to cell chloride concentration in depleted ascites cells (see Methods) is comparatively low, but with undepleted cells once washed it is about 2·2; the chloride ratio is thus a good deal higher than that of human erythrocytes, which is about 1·4. Since the protein content of the ascites cell (as judged by its water content) is only about half that of the erythrocyte, it must be presumed that the greater asymmetry of the former is due to a higher content of phosphoric esters.

The concentration of Na + K in the actively metabolizing ascites cell is about 12 % greater than that of the external medium, and is qualitatively similar, though quantitatively less than Whittam & Davies' figures (1954) for tissue slices. The cation concentration of fresh ascites cells is very variable, but a typical example of ionic ratios is the following: $[K_i]:[K_e] = 100:8 = 12{\cdot}5$; $[Na_i]:[Na_e] = 40:140 = 0{\cdot}29$; $[Cl_e]:[Cl_i] = 130:65 = 2{\cdot}0$, from which it follows that the transport of Na and that of K, though possibly linked, are both active, and that one is not moved passively as a result of potentials set up by movements of the other (cf. Harris & Maizels, 1952, on the human erythrocyte).

Rate coefficients have been measured by the 'chemical method' of Harris & Maizels (1951), because owing to the speed of cation exchange ordinary tracer methods are not applicable. Results, more especially in the presence of metabolic poisons, are apt to be quantitatively inexact, and it is probable that a flow-meter (Tosteson, 1956) suitably slowed would give more accurate and informative results. In the absence of such a device, the chemical method, based on rate of fall of cell Na concentration in ascites cells charged with Na, has given values for the rate coefficient of Na efflux between 3 and 18/hr, and for the coefficient of influx 1–4 and usually 2–4/hr; this corresponds to a Na exchange of about 300–600 m-equiv/l. cells.hr, when the cells are at equilibrium with the external medium, though the *initial* efflux on incubating Na-charged cells may be as great as 1200 or 1400 m-equiv/l. cells.hr. This is 60–120 times faster than the exchange rate of an equal volume of erythrocytes, probably several times faster than the exchange rate for leucocytes, as judged by Hempling's (1954) potassium figures, and comparable to the figures for Na turnover in slices of normal tissues. If the Na exchange is expressed in $pmole/cm^2.sec$, the figure for ascites cells is 21–42, compared with 5 for frog muscle (Keynes, 1954) and 0·06 $pmole/cm^2.sec$ for the human erythrocyte (from Harris & Maizels' 1951 data).

SUMMARY

1. Cations penetrate ascites cells very rapidly. The distribution of added radiosodium suggests that all cell Na is exchangeable, but the movement of Na* is so fast that the measurement of transfer coefficients by means of tracers is impracticable.

2. A method of measuring the coefficients by charging the cells with Na at 4° C and following the rate of fall of cell sodium concentration is discussed. The rate coefficient for Na efflux commonly varies between 4 and 14/hr, and of influx between 2 and 4/hr.

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THE EFFECTS OF CERTAIN PHYSICAL FACTORS AND OF THE CARDIAC GLYCOSIDES ON SODIUM TRANSFER BY MOUSE ASCITES TUMOUR CELLS

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This paper is concerned with the effects of various physical agencies and of the cardiac glycosides on sodium transfer by mouse ascites tumour cells. Since the exchange of Na and radiosodium is very rapid, it was impracticable to measure transfer coefficients by means of tracers, and a 'chemical method' (Harris & Maizels, 1951) has been used in which the cell Na concentration ($[Na_i]$) is first raised by keeping a cell suspension at 4° C in K-free media, and the fall of $[Na_i]$ on incubation at 35° C with K is then observed. k_2 and k_1, the rate coefficients for Na efflux and influx are calculated from the equations

$$k_2t = \ln\{([Na_i]_\infty - [Na_i]_0)/[Na_i]_\infty - [Na_i]_t)\}$$

and

$$k_1 = k_2[Na_i]_\infty/[Na_e],$$

where $[Na_i]_0$, $[Na_i]_t$ and $[Na_i]_\infty$ are the cell Na concentrations at times '0', 't' and '∞' (for details see Maizels, Remington & Truscoe, 1958*a*). Suspensions contained only 2% (v/v) of cells, so it may be assumed that the concentrations of external cations were unaffected by changes in the cell cation concentrations. It should be noted that cell concentrations are expressed in m-equiv/l. cell water, the contributions of the intercellular medium to cation and water in the packed cells being deducted.

METHODS

Cells were usually suspended in a medium containing 1·75 mM-$CaCl_2$, 1 mM-$MgCl_2$, 12·5 mM-Na succinate and 25 mM-glucose, with human serum albumin 3 g/100 ml. and phenol red 1·75 mg/100 ml.; the purpose of the latter was to help in the control of shift in pH due to glycolysis. 130 mM-NaCl and 8 mM-KCl were added to the standard medium, but in the case of other media Na and K were added to suit requirements; for general details see Maizels *et al.* (1958*a*); modifications are described in the text below.

RESULTS

Effects of cell volume on Na transfer

Since in some metabolic experiments the preliminary treatment of ascites cells is such that they are shrunken at the beginning of the actual period of observation, the cell volume returning to normal after 10 min at 35° C, it was necessary to obtain some idea of the effect of cell volume on Na transfer.

A washed and chilled suspension of ascites cells was centrifuged, and equal amounts of a concentrated cell suspension added to the standard medium (see Methods) and to a similar medium containing sucrose. Cells in 'sucrose' suspensions were about 20% shrunken, and so remained during 30 min incubation at 35° C. In one experiment with 3·5% (w/v) sucrose the control and 'sucrose' cell volumes were 100 and 78% of the standard volume, the rate coefficient for Na efflux (k_2) (uncorrected for changes in cell volume) was respectively 12·7 and 9·9/hr, the rate coefficients for Na influx (k_1) were 3·3 and 3·1/hr, while the respective ratios of cell and external Na + K concentrations, $([\mathrm{Na}_1]+[\mathrm{K}_1]):([\mathrm{Na}_e]+[\mathrm{K}_e])$, were 1·12 and 1·24. In a second experiment with 3% (w/v) sucrose the control and 'sucrose' cell volumes were 100 and 82% of the standard volume, k_2 was 11·8 and 10·3/hr, k_1 was 4·0 and 3·8/hr and the Na + K concentration ratios (cells: external medium) were respectively 1·15 and 1·32. Hence when ascites cells were caused to shrink by the addition of sucrose, k_2 appeared to decrease by 10–20%, while the concentration of cell base (as represented by $[\mathrm{Na}_1]+[\mathrm{K}_1]$) definitely increased.

Effects of pH *on rate coefficients*

On the whole changes in pH between 7·2 and 7·5 (approx.) had little effect on k_2, but probably increased k_1 slightly (Table 1), for $[\mathrm{Na}_1]$ at 30 min shows a definite increase when the pH is raised. Respiration decreased by about

TABLE 1. Effect of pH on the rate of Na transfer by ascites cells at 35° C

No.	Type of preparation	pH at 20° C	$[\mathrm{Na}_1]$ at 0 min	$[\mathrm{Na}_1]$ at 30 min	k_2 (hr^{-1})	k_1 (hr^{-1})	Glucose (μmole/ml. cells.hr) assumed converted to	
							CO_2	Lactate
1	Undepleted	7·37–7·41	94	37	10·1	2·5	10·4	80
		7·48–7·55	94	36	10·0	2·4	9·4	95
2	Undepleted	7·15–7·20	68	33	11·1	2·2	8·7	60
		7·28–7·39	72	37	11·8	2·7	—	—
		7·45–7·54	71	39	11·2	2·7	7·7	85
3	Depleted	7·23–7·33	177	101	8·5	5·1	10·8	120
		7·38–7·43	174	101	8·3	5·1	—	—
		7·47–7·53	177	114	7·9	5·7	9·2	140

$[\mathrm{Na}_1]$ is the concentration of cell Na (m-equiv/l. cell water). k_2 and k_1 are the rate coefficients for Na efflux and influx. The cells were incubated in the standard medium.

The respective contributions of respiration and glycolysis are more easily compared if expressed in terms of glucose from which they are presumed to derive, though a proportion of CO_2 is probably not derived from glucose. Depleted cells have been subjected to incubation for 1 hr before the experiments proper were begun.

15 % for a rise in pH of about 0·25, while glycolysis rose by 20–40 %. Assuming that it is glucose that is being metabolized, then the change of glycolysis with pH is considerable; respiration, on the other hand, varies inversely with pH, but the effect is small.

TABLE 2. Effects of temperature on the rate of Na transfer, respiration and aerobic glycolysis of ascites cells

No.	Type of preparation	Temp. (° C)	$[Na_1]$ (m-equiv/l. cell water) at 0 min	60 min	$t=\infty$	k_2 (hr^{-1})	k_1 (hr^{-1})	Glucose (μmole/ml. cells.hr) assumed converted to CO_2	Lactate
1a	Undepleted	25	86	30	29	5·5	1·1	3·3	102
b	Undepleted	35	84	30	30	13·4	2·8	9·1	213
2*a*	Undepleted	25	49	24	23	3·0	0·46	2·4	21
b	Undepleted	35	48	26	26	7·5	1·3	7·1	61
3*a*	Depleted	25	143	58	50	3·1	1·1	4·3	43
b	Depleted	35	136	63	63	10·1	4·5	11·2	121
4*a*	Undepleted	25	94	32	30	2·9	0·62	3·3	33
b	Undepleted	35	91	37	37	8·6	2·2	8·7	81
c	Depleted	25	137	43	42	2·1	0·62	3·2	25
d	Depleted	35	134	46	46	6·7	2·2	9·0	60

For explanation see Table 1

TABLE 3. Temperature coefficients (25–35° C) for the rates of Na transfer, respiration and aerobic glycolysis of ascites cells. Data from previous table

No.	Type of preparation	k_2 Q_{10}	k_1 Q_{10}	Respiration Q_{10}	Aerobic glycolysis Q_{10}
1	Undepleted	2·4	2·5	2·7	2·1
2	Undepleted	2·5	2·8	2·9	2·9
3	Depleted	3·3	4·0	2·6	2·8
4	Undepleted	3·0	3·6	2·6	2·5
	Depleted	3·2	3·6	2·8	2·4

Effects of temperature on rate coefficients

Table 2 shows that respiration, aerobic glycolysis and the rate coefficients for Na efflux and influx (k_2 and k_1) all increase with rise of temperature, Q_{10} being roughly the same for all (Table 3), and usually varying between 2·5 and 3·6. As a rule Q_{10} for k_1 is greater than that of k_2; this is an expression of the fact that $[Na_1]_\infty$ (and also $[Na_1]$ at 60 min) is lower at 25° C than at 35° C, and in conformity values for $[K_1]$ (not shown in the table) are higher at 25° C than at 35° C. It will be realized, of course, that at some temperature below 25° C conditions are reversed, and the rate of efflux decreases relatively more than that of influx, so that $[Na_1]$ again increases; the point of reversal has not been determined. Table 3 does not suggest that depleted cells are more affected by temperature than undepleted cells, nor does it suggest that either k_2 or k_1 are more closely correlated with glycolysis than with respiration.

Effects of the initial cell Na concentration ($[Na_1]_0$) *on Na transfer*

Samples of cells were kept at 4° C in media which were standard, except that Na and K were present in varying proportions. In this way the cell concentrations of Na and K were varied. After 30 min, suspensions were gently centrifuged, each deposit taken up in an equal volume of the standard K-free medium, and then rapidly mixed with 48 vol. of the same medium containing K (8 m-equiv/l.) at 35° C, samples being withdrawn for analysis at intervals. Table 4 shows that k_2 is approximately the same whatever the initial value of [Na_i], and hence Na efflux must be proportional to [Na_i]. k_1 and hence Na influx are approximately constant and with variable and decreasing efflux and constant influx [Na_i] falls to the same constant value ([Na_i]$_\infty$) whatever the initial value of [Na_i] (Table 4 and Fig. 1).

TABLE 4. Effects of the initial cell Na concentration on the rate coefficients for Na efflux and influx (k_2 and k_1). Temp. 35° C. External Na concentration, 140–150 m-equiv/l.

No.	[Na_i] at 0 min (m-equiv/l.)	[Na_i] at 60 min (m-equiv/l.)	k_2 (hr^{-1})	k_1 (hr^{-1})
1*a*	85	30	4·3	0·88
b	110	31	4·2	0·88
c	135	31	4·0	0·84
2*a*	45	42	?	?
b	63	42	4·5	1·4
c	96	45	4·8	1·6
3*a*	64	48	?15	?4
b	72	49	14·3	4·2
c	103	51	14·0	4·3

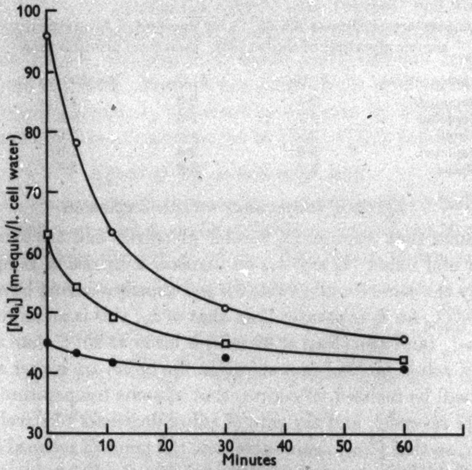

Fig. 1. Effects of the initial cell Na concentration, [Na_i]$_0$, on the fall of [Na_i] in Na-rich cells at 38° C.

Effects of the external Na concentration ($[Na_e]$) *on the rate coefficients for Na efflux* (k_2) *and influx* (k_1)

Equal amounts of a concentrated suspension of ascites cells charged with Na were added to warmed medium containing K in flasks. The medium was standard, except that the amount of NaCl present was varied, tonicity being maintained by the addition of choline chloride. It will be seen (Table 5) that k_2 probably decreases slightly with low $[Na_e]$, k_1 is possibly unaffected, while the ratio $[Na_i]_\infty:[Na_e]$ rises slightly. To what extent these small changes are due to external choline chloride is unknown.

TABLE 5. Effects of the external sodium concentration on the rate coefficients for Na efflux (k_2) and influx (k_1) of ascites cells. External potassium concentration 8 m-equiv/l. Temp 35°C

No.	$[Na_e]$ (m-equiv/l.)	$[Na_i]_\infty$ (m-equiv/l.)	$[Na_i]_\infty:[Na_e]$	k_2 (hr^{-1})	k_1 (hr^{-1})	Na fluxes at equilibrium (m-equiv/l. cell water.hr)
1*a*	145	49	0·34	11·8	4·0	580
b	110	42	0·38	11·7	4·5	490
c	75	28·5	0·38	12·0	4·6	340
2*a*	150	65	0·43	9·1	3·9	590
b	70	34	0·49	7·9	3·9	275
3*a*	142	48	0·34	12·2	4·1	590
b	67	25	0·37	11·0	4·1	270
4*a*	152	49	0·32	14·4	4·6	700
b	71	23	0·32	12·8	4·1	290
5*a*	150	60	0·40	18·7	7·4	1120
b	110	48	0·44	17·6	7·7	850
c	78	36	0·46	17·0	7·9	620

Effects of the external potassium concentration ($[K_e]$) *on the rate coefficients for Na efflux and influx* (k_2 *and* k_1)

Table 6 and Fig. 2 show that with $[K_e]$ as low as 1 m-equiv/l. medium, active transport continues, but k_2 increases progressively as $[K_e]$ is raised to 4 m-equiv, and there is perhaps a further increase in k_2 when $[K_e]$ is raised to about 8 m-equiv/l. medium, beyond which it remains roughly constant. With $[K_e]$ about 0·6 m-equiv/l., $[Na_i]$ so far from falling actually increases, presumably from fall of k_2; the data in these circumstances do not allow either k_2 or k_1 to be calculated. It will be noted that when $[K_e]$ is low, the cells shrink on incubation (Table 6, nos. 3*a*, 4*a* and Table 7, nos. (1*a*, 2*a*) owing to absolute loss of K exceeding absolute gain of Na. With $[K_e]$ between 1 and 15 m-equiv/l. medium, k_1 is roughly constant, as is Na influx measured with $[Na_i]$ at equilibrium ($[Na_i]_\infty$).

Absolute changes in cell Na and K and in cell volume

According to the method used for charging ascites cells with Na, the volume of the cells may remain unaltered, or the cells may shrink or swell (Tables 6–8), and subsequent changes in volume on incubation affect, or are affected by,

TABLE 6. Effects of the external potassium concentration on the rate coefficients for Na efflux and influx (k_2 and k_1) of ascites cells. Temp. 35–38° C. External sodium concentration 145–165 m-equiv/l.

No.	$[K_e]$ (m-equiv/l.)	Cell volume as a percentage of the standard volume at		$[Na_i]$ (m-equiv/l.) at		k_2 (hr^{-1})	k_1 (hr^{-1})	Na fluxes at equilibrium (m-equiv/l. cell water.hr)
		0 min	30 min	0 min	30 min			
1*a*	0·93	95	99	62	47	3·7	1·1	160
b	2·17	95	104	62	34	5·1	1·1	150
c	4·00	95	104	62	30	6·9	1·4	200
d	7·50	95	104	62	28	7·6	1·5	210
2*a*	1·07	93	94	137	112	7·9	5·7	890
b	1·98	93	102	137	90	9·9	5·8	900
c	4·10	93	104	140	76	10·8	5·3	840
3*a*	0·65	104	87	87	111	?	?	?
b	4·2	104	100	87	42	7·0	1·7	270
c	8·8	104	100	87	39	7·6	1·7	270
d	15·7	104	101	87	39	7·8	1·8	290
4*a*	0·52	90	81	71	112	?	?	?
b	4·2	90	98	71	33	9·1	1·9	310
c	8·4	90	100	71	31	10·6	1·9	320
d	15·6	90	102	71	31	10·9	2·0	330

Medium as in previous tables, except that $[K_e]$ is varied. Note: the fluxes are calculated from $[Na_i]_\infty$, not $[Na_i]$ at 30 min; at $t=\infty$, influx and efflux are equal.

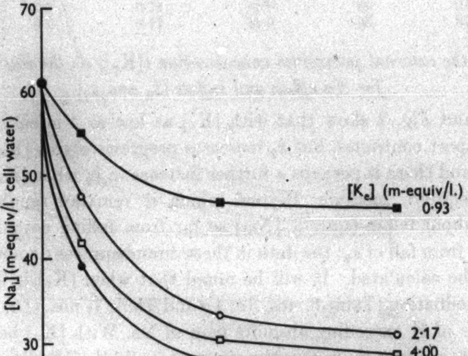

Fig. 2. Effects of the external K concentration ($[K_e]$) on the fall in Na concentration $[Na_i]$ in Na-rich cells at 38° C.

the movements of ions. It is therefore necessary to consider the ways of effecting initial changes in cell volume, and also the results of subsequent incubation, with and without K.

Cells normal in size enriched with Na. This is only applicable to fluids that are not blood-stained. The fluid is then merely cooled to lower metabolism. As a result $[Na_i]$ rises, $[K_i]$ falls and there may be a slight increase in volume. The cold suspension is centrifuged, the cells taken up in a small volume of the K-free medium, and equal volumes of the suspension added to warm samples of the same medium containing varying amounts of added K.

TABLE 7. Changes in cell volume and absolute changes in cell Na and K of ascites cells. Temp. 35–38° C

		Cell volume as a percentage of the standard cell volume at		Absolute changes in Na, K and Na + K (m-equiv/l. standard cells) at					
				0–10 min			10–30 min		
No.	$[K_e]$ (m-equiv/l.)	0 min	10 min	Na	K	Na + K	Na	K	Na + K
1*a*	0·65	104	90	+ 3	− 14	− 11	+ 10	− 25	− 15
b	4·2	104	99	− 30	+ 27	− 3	− 9	+ 7	− 2
c	8·8	104	99	− 32	+ 32	0	− 5	+ 11	+ 6
d	15·7	104	101	− 36	+ 39	+ 3	− 5	+ 11	+ 6
2*a*	0·52	90	83	− 2	− 29	− 31	+ 23	− 37	− 14
b	4·2	90	95	− 15	+ 19	+ 4	− 4	0	− 4
c	8·4	90	98	− 19	+ 35	+ 16	+ 1	− 2	− 1
d	15·6	90	100	− 19	+ 38	+ 19	− 1	− 3	− 4
3*a*	0·19	156	154	− 2	0	− 2	− 2	0	− 2
b	6·3	136	101	− 82	+ 26	− 56	− 20	+ 20	0
4*a*	5·5	139	102	− 88	+ 26	− 62	− 38	+ 34	− 4
b	5·5	78	92	− 12	+ 27	+ 25	− 8	+ 14	+ 6
5*a*	8·0	114	102	− 53	+ 35	− 18	− 20	+ 22	+ 2
b	8·0	93	97	− 27	+ 34	+ 7	− 16	+ 19	+ 3
6*a*	8·2	111	100	− 29	+ 14	− 15	− 10	+ 9	− 1
b	8·2	89	100	− 9	+ 22	+ 13	− 10	+ 8	− 2

Expts. 1 and 2 are the same as expts. 3 and 4 of the previous table. Note: volumes in previous table at 0 and 30 min; in above table at 0 and 10 min.

On incubation with medium containing K, cell volume alters but little; fall of Na equals gain of K, suggesting a 1:1 linkage for transfer (Table 7, nos. 1*b*, *c*, *d*). If, on the other hand, the cells are incubated in a nutrient medium containing no K, cell Na concentration ($[Na_i]$) rises (Table 6, nos. 3*a*, 4*a*), while $[K_i]$ (not shown in the table) falls. The fluxes, however, are not linked, for the records of this same case in Table 7 (no. 1*a*) show that absolute gain in Na (i.e. change in Na/l. standard cells or alternatively change in Na/cell) is slight, while absolute loss of K is considerable; hence the cell loses water and shrinks. The findings arise as follows: as a result of the preliminary treatment, the ascites cell has lost K, but a significant amount still remains, and further loss of K and shrinkage during incubation depend on the steep concentration gradient, from perhaps 60–80 m-equiv K/l. cell water to 0 m-equiv in the external medium; this unlinked loss of K is presumably passive and must occur in combination with anion.

Cells shrunken and enriched with Na. The method of achieving this follows from what has just been said. In practice it was combined with a procedure for ridding the ascites fluid of erythrocytes (see Methods: Maizels *et al.* 1958*a*) The fluid, mixed with about 10 vol. of NaCl solution, was gently centrifuged and the ascites cells resuspended in 20 vol. of cold K-free medium. At this stage cell K is reduced but a fair amount still remains, and on warming the steep gradient from cell to external K concentration together with the relatively gentle gradient from external to cell Na concentration determines an absolute decrease in cell cation and volume.

If cells so shrunken are transferred to a fresh batch of K-free medium, they may shrink still further, from loss of residual K exceeding gain of Na (Table 6, 4*a*; Table 7, 2*a*). If instead the shrunken cells are incubated with a nutrient medium containing K, absolute gain of K exceeds absolute loss of Na, and the cell volume tends to return to normal.

Cells swollen and enriched with Na. This was achieved by suspending the cells in 100 vol. of cold K-free NaCl solution, centrifuging and transferring to a second change of NaCl solution, and finally transferring to cold K-free medium before incubation. During the course of this treatment there may be a brief period of shrinking, but ultimately the cells are denuded of K, so that their composition approximates to that of the external medium, and as a result of their content of non-penetrating anion, Na and water enter the cells, which swell. On incubation with a K-free medium, little further change in volume or in composition occurs: thus in Expt. 3*a* (Table 7) cell volume at the beginning of incubation was 156 %, [Na_i] was 161 m-equiv and [K_i] 2 m-equiv/l. cell water; at 10 min (and also at 30 min) all these values were practically unchanged. If, however, the K-depleted swollen cells are incubated with K, there is a great absolute loss of Na, a moderate increase in cell K with a large absolute decrease in Na+K and volume (Table 7, no. 3*b*). The experiments thus suggest that the effect of incubation in the presence of K is to restore cell volume to normal, irrespective of the initial volume. To test this, Expts. 4–6 of Table 7 were devised. In Expts. 1–3 of this table, the initial volumes of cells were all the same, and alteration of volume during incubation was effected by varying the amount of K added. In Expts. 4–6 of Table 7, cell volume was varied by methods already described, so that the cells were either swollen or shrunken at the beginning of incubation. The *same* amounts of K were then added to all suspensions. In the case of the shrunken cells, gain of K exceeded loss of Na and the cells swelled, while with swollen cells gain of K was less than loss of Na and the cells shrank, the ultimate volume being the same in either case. In these and in other experiments, the unequal exchanges of Na and K and the accompanying volume changes were all complete in under 10 min; thereafter cell volume remained constant and the residual Na and K exchanges, which were not inconsiderable, proceeded on a 1:1 basis (Table 7, nos. 1–6).

Changes in the concentration of Na + K and of chloride were also studied. (Table 8), and it was found that convergence of cell volume was accompanied by convergence of the ratio $[\mathrm{Cl_e}]:[\mathrm{Cl_i}]$ (i.e. of the external and cell chloride concentrations) which was initially low in the swollen cells and high in the shrunken cells (Table 8, nos. 2–4). It will also be noted that the respective losses and gains of total base (Na + K) in the case of swollen and shrunken cells are roughly covered by the corresponding changes in chloride, though the correspondence is not quite as good as the figures suggest, because other anions including succinate and lactate will move with the chloride ions, and the figures for change in total anion will be about 15% greater than for $\mathrm{Cl^-}$.

TABLE 8. Ascites cells swollen or shrunken by preliminary treatment and then incubated in media of constant K concentration: comparison of changes in cell volume with absolute changes in Na + K and Cl of the cells; also with changes in the ratio of external and cell chloride concentration, and with changes in the concentrations of Na + K in the cells and external medium. Temp. 35° C

		Cell volume as a percentage of the standard cell volume at		Absolute changes in Na + K and in chloride (m-equiv/l. standard cells) at 0–30 min				$\frac{[\mathrm{Cl_e}]}{[\mathrm{Cl_i}]}$		$\frac{[\mathrm{Na_i}]+[\mathrm{K_i}]}{[\mathrm{Na_e}]+[\mathrm{K_e}]}$	
No.	$[\mathrm{K_e}]$	0 min	30 min	Na	K	Na + K	Cl	0 min	30 min	0 min	30 min
1*a*	5·5	139	100	−126	+60	−66	—	—	—	1·11	1·18
b	5·5	78	99	−20	+51	+31	—	—	—	1·26	1·19
2*a*	8·0	114	100	−73	+57	−16	−16	1·56	1·63	1·09	1·14
b	8·0	93	100	−43	+53	+10	+12	1·80	1·65	1·15	1·12
3*a*	8·2	111	100	−39	+23	−16	−13	1·38	1·46	1·08	1·14
b	8·2	89	100	−19	+30	+11	+11	1·53	1·47	1·17	1·13
4*a*	7·3	168	106	−147	+56	−91	−81	1·26	1·38	1·07	1·19
b	7·3	93	100	−65	+80	+15	+8	1·50	1·48	1·18	1·21
c	7·3	78	100	−43	+72	+29	+26	1·75	1·56	1·31	1·19

$[\mathrm{K_e}]$ = external potassium concentration (m-equiv/l. water);

$\frac{[\mathrm{Cl_e}]}{[\mathrm{Cl_i}]}$ = the ratio of the external and cell chloride concentrations;

$\frac{[\mathrm{Na_i}]+[\mathrm{K_i}]}{[\mathrm{Na_e}]+[\mathrm{K_e}]}$ = the ratio of the cell Na + K concentrations and the external Na + K concentrations.

Note: nos. 1–3 of this table are the same as nos. 4–6 of Table 7, with additional data. No. 4 is a new experiment.

Table 8 also shows that the concentration of Na + K, ($[\mathrm{Na_i}]+[\mathrm{K_i}]$), in the cell water is high in the initially shrunken cells, low in those initially swollen, and converges on an intermediate value during incubation with K, as cell volume (and cell water) return to normal. This conclusion follows from the last column of Table 8, for since the volume of the external phase is very large, $[\mathrm{Na_e}]+[\mathrm{K_e}]$ may be regarded as constant, and hence $[\mathrm{Na_i}]+[\mathrm{K_i}]$ varies directly

TABLE 9. Effects of the cardiac glycosides on the Na transfer of ascites cells. Temp. 35° C. Medium as in previous tables. $[Na_e]$ 140–150 m-equiv/l., $[K_e]$ 8 m-equiv/l.

No.	Cardiac glycoside (μg/ml.)	$[Na_i]$ (m-equiv/l. cell water) at		k_2 (hr^{-1})	k_1 (hr^{-1})	Na fluxes at equilibrium (m-equiv/l. cell water.hr)	Glucose (μmole/ml. cells.hr) assumed converted to	
		0 min	40 min				CO_2	Lactate
1*a*	Digoxin 0	89	40	12·2	3·4	490	8·2	86
b	Digoxin 4	97	40	8·0	2·2	320	—	—
c	Digoxin 10	101	42	4·6	1·3	190	—	—
d	Digoxin 40	118	54	3·3	1·0	?140	8·2	87
2*a*	Strophanthin G 0	72	51	4·5	1·5	220	8·1	64
b	Strophanthin G 1	72	51	3·7	1·2	180	—	—
c	Strophanthin G 10	77	51	2·6	0·85	120	8·7	62
d	Strophanthin G 100	88	82	?	?	?	7·9	61
3*a*	Strophanthin G 0	136	43	4·8	1·3	200	—	—
b	Strophanthin G 5	133	45	3·8	1·0	150	—	—
c	Strophanthin G 20	134	55	2·9	0·85	120	—	—
d	Strophanthin G 80	144	85	?1·9	?0·65	?90	—	—
4*a*	Strophanthin G 0	116	62	8·5	3·6	520	—	—
b	Strophanthin G 5	122	67	5·2	2·4	350	—	—
c	Strophanthin G 20	133	74	3·2	1·4	200	—	—
d	Strophanthin G 60	138	88	?2·2	?1·1	?160	—	—

Symbols as in previous tables. There is a wide margin of error in 2*d*, 3*d* and 4*d* because of uncertainty in extrapolation to $t = \infty$.

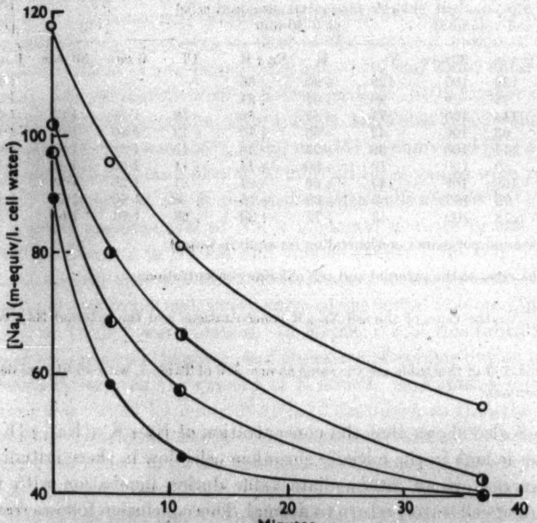

Fig. 3. Effect of digoxin on the fall of Na concentration $[Na_i]$ in Na-rich cells. Temp. 35° C. ○, 40μg/ml.; ◑, 10μg/ml.; ◐, 4μg/ml.; ●, 0μg/ml.

with ($[Na_i]+[K_i]$):($[Na_e]+[K_e]$). Hence as swollen cells shrink, total base is absolutely decreased but the concentration of base rises, while as shrunken cells swell, total base is absolutely increased but the base concentration falls.

Effects of the external potassium concentration on metabolism

Marked reduction in $[K_e]$ was associated with marked decrease in glycolysis and a rather small decrease in respiration. Thus with $[K_e]$ at 0·2 m-equiv/l. medium, 9·3 μmole glucose/ml. cells.hr was converted to CO_2 and 31 μmole to lactate, while with $[K_e]$ at 4·2 m-equiv the figures for CO_2 and lactate were 11·6 and 112. In Expt. 3 of Table 7 with $[K_e]$ at 0·19 m-equiv/l. medium, respiration and glycolysis corresponded to 7·3 and 6·5 μmole glucose utilized, while with $[K_e]$ at 6·3 m-equiv, the figures were 9·5 and 45 μmole/ml. cells.hr.

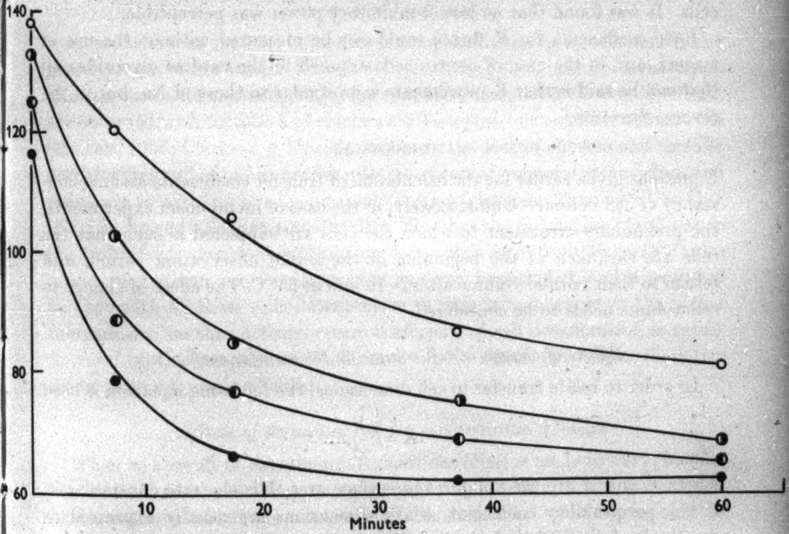

Fig. 4. Effect of strophanthin G on the fall of Na concentration $[Na_i]$ in Na-rich cells. Temp. 35° C. ○, 60 μg/ml.; ◑, 20 μg/ml.; ◐, 5 μg/ml.; ●, 0 μg/ml.

The cardiac glycosides

Schatzmann (1953) has shown that these prevent active movements of Na and K without affecting metabolism. This is confirmed in Table 9, which also shows that the cardiac glycosides, digoxin and strophanthin G (ouabaine) greatly reduce the rate coefficient for Na efflux (k_2). It will be seen, however, that the level to which $[Na_i]$ falls on incubation with potassium remains fairly constant, unless the concentration of glycoside is very high, and even here (nos. 1*d*, 2*d* and 3*d*) extrapolation to $t=\infty$ gives lower values for $[\text{Na}_i]$ than the 40 min figures shown (Figs. 3, 4). Since, then, unless the external concentration of glycoside is very high $[\text{Na}_i]_\infty$ falls to the same concentration on incubation with K, while k_2 decreases with quite moderate amounts of glycoside, it follows that both Na efflux and influx must show a corresponding decrease in accordance with the equation $k_2[\text{Na}_i]_\infty=k_1[\text{Na}_e]$. The possibility was considered that the glycosides were gradually destroyed by the ascites cells, which recovered from the effects of poisoning as incubation proceeded, so that fall of $[\text{Na}_i]$ was at first slow, but later during incubation became normal. To test this, ascites cells were incubated with various media containing enough strophanthin G to give 0, 30 and 60% decrease in k_2. After 30 min the suspensions were centrifuged and the supernatants incubated with fresh ascites cells. It was found that no loss of inhibitory power was perceptible.

Rate coefficients for K fluxes could not be measured without the use of tracers, and in the case of ascites cells exposed to the cardiac glycosides all that can be said is that K movements were similar to those of Na, but in the reverse direction.

DISCUSSION

Equations given earlier for the calculation of transfer coefficients assume constancy of cell volume. Unfortunately, in the case of metabolism experiments, the preliminary treatment to which the cells are subjected is such that the cells are shrunken at the beginning of the actual observation period, and return to their normal volume after 5–10 min at 35° C. The effect of change in cell volume needs to be considered.

Effects of change in cell volume on the transfer coefficients

In order to relate transfer to cell dimensions, the following equation is used

$$K=k\frac{V}{A},$$

where V and A are the volume and surface area, k is the rate constant and K the permeability coefficient, whose dimensions are usually expressed as cm.sec^{-1}. Assuming that the equation or a modification of it holds for an actively transporting system and that K is in fact constant with change in cell volume, then with decrease in cell volume, k should increase. It has been seen, however, that with a decrease in cell size of 20%, k_2 so far from being greater, is actually 10 or 15% less than k_2 of the control, and if the sucrose itself has no adverse effect on the cells, then decrease of k_2 suggests that, as a result of shrinking, areas for active transport are rendered inaccessible to cation: this might arise if the membrane were wrinkled in such a way as to make the walls of the wrinkle adhere, and seal a proportion of the transporting channels.

However this may be, the findings indicate the futility of attempting to correct k_2 for changes in cell volume during incubation, and it has been necessary to use only the following experiments: (1) experiments where the change in cell volume is less than 10%; (2) experiments where the main changes in volume are completed between 0 and 5 min, and where the 5–40 min curve provides an adequate fall of $[\mathrm{Na}_i]$; (3) experiments where change in volume is less than 15%, and where the several suspensions, though treated differently, all show the same volume changes. This is in fact usually the case, for the 'chemical method' based on fall of $[\mathrm{Na}_i]$ requires that if active transport be inhibited, the inhibition should be incomplete; hence even in suspensions containing metabolic poisons the volume changes are similar to those of the unpoisoned controls. In these circumstances the rate coefficients have a relative rather than an absolute value.

Temperature coefficients

It has been seen that the influences of temperature on k_2, k_1, respiration and glycolysis are rather similar, though between 25 and 35° C influx is rather more affected than efflux; hence $[\mathrm{Na}_i]$ is lower at 25° C than at 35° C. Similar effects are observed with tortoise and chicken erythrocytes, but not with human cells (Maizels, 1956; Clarkson & Maizels, 1955). In the case of chicken and tortoise erythrocytes and of mouse ascites cells the effects of temperature on efflux and influx are reversed at some point (undetermined) between 25 and 4° C, so that $[\mathrm{Na}_i]$ no longer falls, but actually rises. Whether the fall in $[\mathrm{Na}_i]$ (and rise in $[\mathrm{K}_i]$) observed when temperatures are lowered *in vitro* from 35 to 25° C also occurs *in vivo*, can only be determined by direct experiment, and it may well be that with all these cells deterioration *in vitro* is more rapid at the higher temperature, so that a temperature coefficient of cell deterioration is superimposed on the temperature coefficients for Na efflux and influx, the latter being more adversely affected.

Effects of the initial cell Na concentration ($[\mathrm{Na}_i]_0$)

When as a result of experimental conditions $[\mathrm{Na}_i]$ is made to vary, incubation with K results in $[\mathrm{Na}_i]$ of the several samples falling to the same equilibrium values at about the same rates (Table 4 and Fig. 1). It is possible that more refined methods might show some deviation, but the findings justify the assumption that efflux approximates to a first-order process.

Effects of the external Na concentration ($[\mathrm{Na}_e]$)

Glynn (1956) has shown in the case of human erythrocytes that if $[\mathrm{Na}_e]$ is halved, Na influx falls by only 40%; if the effects of choline added to maintain external tonicity can be ignored, this suggests that Na influx is not a first-order process. In the present experiments, similarly conducted, reduction in influx is 0–10% less than the corresponding reduction induced in $[\mathrm{Na}_e]$,

k_2 and k_1 are rather little affected, while the ratio $[Na_i]_\infty : [Na_e]$ rises by 0–15% (Table 5). In view of the unphysiological nature of the choline chloride added, the findings are rather in favour of a first-order process than otherwise. Perhaps deviations may be detected and correlated by more refined methods, but even so it is thought that the assumption of a first-order process for influx, though perhaps approximate, suffices for the present purpose. In any case as long as $[Na_e]$ is always about the same (150 m-equiv/l.), and as long as the rate of influx is unaffected by $[Na_i]$, the equation for k_2 given at the beginning of this paper is still valid.

Effects of the external K concentration ($[K_e]$) *on Na transfer*

As $[K_e]$ is reduced from a high level to some value between 4 and 8 m-equiv/l., k_2 and k_1 remain almost constant, though at the lower value there is a slight increase in $[Na_i]_\infty$ (Table 6) and some decrease in $[K_i]_\infty$ (not shown in the table). With $[K_e]$ set experimentally at 2 m-equiv/l., and more markedly at 1 m-equiv/l., k_2 shows a definite decrease while k_1 is little affected. With $[K_e]$ set at about 0·5 m-equiv, fall of $[Na_i]$ is replaced by a rise, and it is not possible to calculate the rate coefficients using the 'chemical method', nor to show whether Na influx is altered.

It has been seen that in K-poor media the respiration of ascites cells may fall by 20% and glycolysis by as much as 80%, and this effect may account in part for the observed failure in Na transport. Nevertheless, in metabolic experiments (Maizels, Remington & Truscoe, 1958*b*) metabolic inhibition by poisons is equally marked, but the values of k_2 and k_1 show that active Na efflux is still occurring, and in such experiments $[Na_i]$ still falls on incubation, either as a result of residual metabolism, or because of energy supplied by energy-rich phosphate bonds. The marked rise in $[Na_i]$ observed in K-poor media suggests that, in addition to the metabolic defect, some linkage of Na and K transport may exist, so that when K influx is limited by lack of K in the external medium, Na efflux tends to fail as well.

Linkage of sodium and potassium in cation transport

That a 1:1 linkage of Na and K transport exists (provided cell volume remains constant) is suggested by those experiments where cells in K-free media are charged with Na which is then expelled on incubation with K (see Table 7, nos. 1*b–d*): it is also suggested by other experiments where change in cell volume occurs, but is nearly complete in 0–10 min, cell volume remaining almost constant thereafter; under these conditions fall of Na and rise of K between 10 and 30 min are approximately equal (Table 7, nos. 2*b–d*; 3*b*; 4*a*, *b*; 5*a*, *b*; 6*a*, *b*). In the second group of experiments (with change in volume during incubation) it has been noted that the exchanges of Na and K are unequal during the initial stages (0–10 min): it follows that during this stage the movements of cations must be accompanied by an appropriate shift of anions, subsequent shifts of cation (20–30 min) being on a 1:1 basis. This indicates that the anion movements reach completion appreciably faster than the cation movements, a finding which might be expected from Tosteson's (1956) demonstration that in the case of human erythrocytes chloride transfer is many thousand times faster than that of cations.

It is now necessary to consider in some detail the response of swollen or shrunken cells to incubation. In the section on Results it was noted that if ascites cells were repeatedly washed in cold NaCl solution they swelled and their composition approximated to that of the external medium. On incubating such cells in a K-free nutrient medium at 35° C, the Donnan asymmetry factor suggests that the cell ought to show an absolute passive increase of 20–40 m-equiv Na/l. cell water.hr and should continue to swell; but though the cell remains swollen, no further increase in cell Na or in volume is observed (Table 7, no. 3*a*), each remaining constant. This suggests the possibility of an independent Na efflux opposing the effects of asymmetry, or alternatively that at 35° C the minute amount of K in the external medium (0·19 m-equiv/l.) could contribute to active transport. When K was added to such a system where both cells and medium are practically K-free, there was in one expt. (Table 8, 4*a*) an absolute fall in Na of 147 m-equiv/l. standard cells, K rising by 56 m-equiv, and the cells shrank by 40% in 30 min. Similar changes, definite but less marked, are seen in Table 8, no. 1 and Table 7, 3*b*. In all these cases a 1:1 linkage is not apparent and the data suggest the possibility of an unlinked active Na efflux, Na being accompanied by anion. Alternatively, it might be suggested that a 1:1 linkage exists, in which case one must assume that there is a considerable passive efflux of the K which has entered actively, in association with the active efflux of Na. One may imagine the process occurring in two stages, the first of active 1:1 exchange of Na and K without reduction in cell swelling, and the second stage in which the accumulated K flows passively from the high cell concentration to the low external concentration, the loss of K then causing the cell to shrink. In fact $[K_e]$ in Expt. 4*a* (Table 8) was 7 m-equiv/l., a level which in most cell suspensions suffices to maintain a high level of cell K. The alternative view thus assumes that either as a result of injury during preparation, or in association with K deprivation, cell permeability to passive penetration has increased. This may well have been the case in Expt. 4*a* (Table 8), where $[Na_i]_\infty$ was higher than that of the parallel suspension of shrunken cells, but in Expts. 1–3 there was no evidence that permeability had increased significantly. Hence the possibility of unlinked Na active efflux is not excluded.

In the case of shrunken cells in K-free media, incubation and the addition of K causes K influx to exceed Na efflux and the cells swell. It may be assumed that shrinkage leads to an unlinked active K influx, or alternatively that the findings arise from the increased Donnan asymmetry of the initially shrunken cell. This will determine an increased passive influx of Na into the cell, which together with the initially high value for [Na_i] will promote an increased active efflux of Na (Fig. 1), and on the basis of a 1:1 Na–K linkage will lead to an increased active influx of K. Thus the tendency to increased passive entry of Na is translated into an increased active entry of K, and so the cell swells.

Reverting to the question of active Na efflux in K-free media: Flynn & Maizels (1949) have observed shrinkage of human erythrocytes on incubation in K-poor media, which might be due to unlinked active Na efflux, or to the concentration gradients leading to loss of K exceeding gain of Na. So, too, Hodgkin & Keynes (1955) have shown that there occurs an efflux of Na from *Sepia* nerve in K-free media, which might, however, have occurred on an exchange basis with a local accumulation of K, itself derived by diffusion from the nerve interior.

The control of cell volume

It is suggested that in the normally metabolizing cell constancy of composition and volume is maintained by a balance of active Na and K fluxes on a 1:1 basis and of passive fluxes subject to the Donnan asymmetry. If metabolism is suspended there may be a temporary phase of shrinkage due to their respective concentration gradients leading to loss of K exceeding gain of Na, but once K equilibrium has been attained the effects of asymmetry become manifest and the cell ultimately swells, taking up NaCl and water from the external medium. As a result the concentration of non-penetrating anion, and the asymmetry, progressively decrease. If now metabolism is initiated by incubating with K and glucose, the active cation fluxes are re-established, and act at an advantage because the asymmetry is low. Hence efflux exceeds influx (actually loss of Na exceeds gain of K), the cell shrinks and the asymmetry increases, the processes proceeding until the normal balance of fluxes and asymmetry is restored.

In the case of the shrunken cell incubated with glucose and K, water content is initially decreased, the concentration of non-penetrating anion is high, and the balance of fluxes and asymmetry is disturbed in favour of the asymmetry factor. As a result cation influx exceeds efflux (gain of K exceeds loss of Na), and the cells swell until equilibrium is restored between the fluxes and the effects of the asymmetry.

The findings accord with the views of Leaf (1956). In the case of the initially swollen cell (Table 8), they may be summarized as follows: (1) water content is higher than in normal cells, (2) the concentration of non-penetrating anion is lower, (3) Na + K per cell is higher, (4) the concentration of Na + K in cell water is lower, (5) [Cl_e]:[Cl_i] is lower. On incubation with glucose and K all these changes tend to be reversed, and, as the cell shrinks, base leaves the cell in combination with anion and accompanied by water. Changes in the shrunken cell, and also in the shrunken cell as it returns to normal on incubation, are the exact reverse of those just described.

It must be emphasized that with an absolute increase of cell base (i.e. base per cell), the cell swells, the concentration of non-penetrating anion decreases and the *concentration* of cell base falls. With an absolute decrease in cell base, the cell shrinks and the concentration of cell base rises. If cells are shrunken by adding sucrose a similar increase in cell base concentration occurs.

Finally, in this connexion it may be noted that volume changes similar to those characteristic of the ascites cells occur in the human erythrocyte, which shrinks in potassium-free media and swells on the addition of K; so too, human erythrocytes which have gained Na and total base during cold-storage, lose base and shrink on incubation with media containing K. Similar changes occur with chicken and tortoise erythrocytes (Maizels, 1954).

The cardiac glycosides

Schatzmann (1953) has shown that the cardiac glycosides inhibit cation transport by erythrocytes, without affecting metabolism (cf. Table 9), and Glynn (1957) attributes this to successful competition with K for carrier sites. In the case of ascites cells, the 'chemical method' does not permit K influx to be measured, but the glycosides obviously decrease the rate coefficient for Na efflux (k_2) and the efflux itself (Table 9 and Figs. 3, 4), and it may be presumed that K influx is also decreased, since at constant cell volume Na and K exchange on a 1:1 basis.

The glycosides also reduce Na influx, though if the concentration of the poison is high, k_2 is more inhibited than k_1 (the rate coefficient for Na influx), and $[Na_i]$ is relatively high (Table 9 and Fig. 4). But with moderate or small amounts of glycoside, though k_2 decreases, $[Na_i]$ on incubation falls to a roughly constant figure for $t=\infty$. Since at equilibrium $k_2\,[Na_i]_\infty = k_1\,[Na_e]$ and $[Na_e]$ is constant, it follows that the inhibitions of Na efflux and influx must be proportional, as must the decreases in k_2 and k_1. The findings with ascites cells thus differ from those of human erythrocytes. In the latter, Glynn (1957) shows that inhibition by glycosides of K influx and Na efflux may exceed 70%, while Na influx is unaffected unless K is absent from the external medium, in which case inhibition occurs. The explanation may lie in the fact that in Glynn's experiments containing both K and glycoside the two were always present together, while in the present experiments it was necessary to equilibrate the ascites cells with glycoside for 15 min before active transport was initiated by the addition of K. During this K-free period, it is possible that the glycoside became more firmly attached to all the carrier sites (active and passive), than would have been the case had glycoside and K been added simultaneously.

Again, Glynn (1957) finds that while the cardiac glycosides do not inhibit Na influx in the presence of external K, with K-free media maximal inhibition amounts to 40 %. The cause of this is obscure, and may perhaps be due to competition for carrier sites. Shaw (1955) has suggested that the increased Na influx occurring with very low [K_e] may be due to the carriage inward of Na by K carriers, which in the absence of external K are left free; if so, blocking by digoxin may prevent this extra Na influx. This explanation could hardly apply to ascites cells, however, for here the reductions of the fluxes and the transfer coefficients are proportional even in the presence of external K, and it seems probable that the glycosides directly block the passive transfer of Na. The possibility was considered that the relative constancy of [Na_i] might be due to detoxication of glycosides by ascites cells, with recovery of the cells as incubation proceeded; this, however, was excluded by direct experiment.

Comparison of the data in Table 9 with Glynn's figures for erythrocytes suggests that ascites cells are perhaps forty times as resistant to the action of the cardiac glycosides; but while in the case of erythrocytes it is mainly active transport which is blocked, in the case of acites tumour cells passive carriers appear to be blocked as well, in which case speculation on the relative number of active carrier sites become irrelevant.

SUMMARY

In the following, k_2 is the rate coefficient for Na efflux, k_1 for Na influx, $[Na_i]_0$ is the initial cell Na concentration (m-equiv/l. cell water), and $[Na_i]_\infty$ is the equilibrium value to which the Na concentration of incubated cells falls at $t=\infty$. [K_e] is the external potassium concentration and [Na_e] the external Na concentration.

1. The temperature coefficients (25–30° C) for respiration, glycolysis, k_1 and k_2 of mouse ascites tumour cells are all fairly similar, varying between 2·4 and 3·4; Q_{10} for k_1 is slightly higher than for k_2; hence influx is more susceptible to temperature changes than efflux, and as the temperature of a suspension is lowered from 35 to 25° C $[Na_i]_\infty$ falls. At some point below 25° C the effects of temperature on the fluxes is reversed, and further cooling raises $[Na_i]_\infty$.

2. When $[Na_i]_0$ in different samples of the same suspension is made to vary, [Na_i] of all samples on incubation with K falls to the same value for $[Na_i]_\infty$, the rate constant being the same for all irrespective of the value of $[Na_i]_0$.

3. Lowering [Na_e] from 150 to 75 m-equiv/l. medium has rather little effect on k_2, k_1 and the ratio [Na_i]:[Na_e], the latter increasing by 0–15 %. Decrease in Na influx is relatively less than the decrease in [Na_e] (about 45 % compared with 50 %).

4. When [K_e] is made to vary in different samples of the same suspension, k_2 remains fairly constant until [K_e] is reduced to about 4 m-equiv/l., when there

is perhaps a slight fall in k_2. The decrease in k_2 is definite when $[K_e]$ is only 1 m-equiv/l., and then the absolute fall to $[Na_i]_\infty$ becomes small and $[Na_i]_\infty$ itself relatively high. With $[K_e]$ set at 0·5 m-equiv/l., $[Na_i]$ no longer falls, but rises, and the method used for measuring k_2 and k_1 becomes inapplicable.

5. As a result of exchanges of cation, anion and water between cells and K-poor media, ascites cells may be made either to swell or to shrink, even though osmotic and pH changes in the external media are absent. If the cells are now incubated with K, swollen cells shrink, losing Na, gaining K, with a net loss of total base, chloride and water, and with an increase in the Donnan asymmetry and in the cell concentration of Na + K. By contrast, initially shrunken cells incubated with K lose Na and gain K, with a net gain of total base, Cl and water, while asymmetry and the concentration of cell Na + K decrease. Thus initially swollen or shrunken cells incubated with K approach the same volume and the same ionic concentrations.

6. The cardiac glycosides depress k_2 and k_1 proportionally, and hence the level to which Na falls at $t = \infty$ is unaffected, though it is attained more slowly than in the control.

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METABOLISM AND SODIUM TRANSFER OF MOUSE ASCITES TUMOUR CELLS

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The present paper is concerned with the effects of metabolism on Na transfer by ascites tumour cells. The rate coefficients for Na transfer have been calculated from the rate of fall of cell Na concentration ($[\mathrm{Na}_i]$) in cells first charged with Na by keeping in a K-free medium, Na transport subsequently being initiated by warming to 35° C and adding K (Maizels, Remington & Truscoe, 1958*a*, *b*). The investigation has presented difficulties because, even in the presence of agents affecting metabolism and depressing active transport, the effects have not been immediate. Thus, in the presence of iodoacetate, after 1 hr incubation the cell Na concentration ($[\mathrm{Na}_i]$) will have fallen from the high initial value to a level which is as low as that of the unpoisoned control, even though some inhibition of glycolysis occurs. Thereafter, $[\mathrm{Na}_i]$ in the unpoisoned suspensions remains low, while that of the poisoned suspensions begins to rise. It is thought that this may be due in part to the presence of intermediate metabolites still capable of being metabolized even in the presence of iodoacetate, or possibly to the mediation of substances with energy-rich phosphate bonds. However this may be, it is necessary to minimize this latent period by incubating ascites cells for about an hour before the curves for fall of $[\mathrm{Na}_i]$ against time are begun. Again the measurement of respiration requires 15 min equilibration before manometric readings are begun, and hence observations on the fall of $[\mathrm{Na}_i]$ ought to be similarly delayed. Ordinarily, however, $[\mathrm{Na}_i]$ would already have approached equilibrium during this preliminary incubation, and to avoid this all the preparatory stages must be conducted in K-free media, since in these circumstances $[\mathrm{Na}_i]$ remains high even at 35° C (Maizels *et al.* 1958*b*).

It should be noted that throughout the following, cell concentrations (i.e. $[\mathrm{Na}_i]$) are given in m-equiv/l. cell water, a deduction having been made for contributions by the intercellular medium.

METHODS

Most of these have been considered earlier (Maizels *et al.* 1958*a*, *b*). When investigating the effects of various substrates, cells must first be deprived of their natural substrates as far as possible, by washing in a large volume of NaCl solution and suspending the cells in dialysed albumin (3%) in NaCl (0·14 M). After 50 min at 35° C, substrate is added, and after a further 10 min the suspension is cooled to 4° C. In 30 min it is centrifuged from the medium, which now contains K leached from the cells, and the latter are resuspended in fresh K-free albumin solution containing substrate. This is equilibrated for 15 min at 35° C, K is added to 8 m-equiv/l. and time curves for fall of $[Na_i]$ are begun.

When investigating the effects of cyanide, the same method was used except that 25 mM glucose and 12·5 mM-Na succinate were present in the final incubation medium, and reservoirs containing appropriate KOH–NaCN (Krebs, 1935) were inserted into the flasks.

The investigation of iodoacetate presented special difficulties because the poison had a cumulative effect, which caused $[Na_i]$ to rise on incubation after a preliminary fall. To minimize this the following procedure was adopted: ascites cells were given the usual preliminary treatment in K-free albumin-NaCl medium, iodoacetate being added after 50 min, and the suspension was cooled on ice 10 min later. After 30 min the cold suspension was centrifuged, and the deposit taken up in fresh K-free medium containing glucose and Na succinate. After a further 30 min in the cold, the suspension was incubated at 35° C for 20 min, KCl was added and observations were begun. Thus iodoacetate was present for only a short while during the preliminary stage, and not at all during the actual period of incubation, it being presumed that those SH– groups combined with iodoacetate would remain inactivated even when the poison was removed. It might be thought that if this presumption were wrong, then with a recovering cell calculation would give a value for k_2 which was in fact too low, just as with a deteriorating cell, k_2 may appear too high (see later). To meet this objection lactate estimations were always done for separate 0–20 and 20–40 min periods. In all cases lactate formed during the second period was the same as, or less than that formed during the first period, showing that no recovery had occurred. It may be noted that the sensitivity of ascites cells to iodoacetate varied greatly, but was always increased by depletion and washing.

RESULTS

Effects of various substrates

In the following text, k_2 is the rate coefficient for Na efflux and k_1 is the rate coefficient for Na influx. It should be noted: (1) that the absence of certain substrates is associated with a marked rise of $[Na_i]$ in the preparatory K-free stages, and hence at the beginning of incubation the several values of $[Na_i]$ are not necessarily the same (Table 1); (2) that the rate coefficients are calculated from the *fall* of $[Na_i]$; hence in the case of the metabolic poisons enough must be added to reduce the rate of fall, but not to abolish the fall altogether.

No substrate added. In the absence of added substrate, and in spite of preliminary washing and incubation, the cells continue to respire for about an hour at a rate which may exceed that of suspensions containing glucose (Table 1); thereafter respiration gradually declines. Aerobic glycolysis is absent, but CO_2 must be derived partly from glucose since, if cyanide be added, reduction in respiration equals 6·5 μmole glucose/ml. cells.hr spared, while extra lactate formed corresponds to 3·2 μmole glucose glycolysed. But there must be other sources of CO_2 besides glucose, for if all CO_2 produced in the absence of cyanide were derived from glucose, the addition of cyanide should give 6·5 μmole glucose glycolysed, instead of only 3·2 μmole/ml. cells.hr observed (Table 1, no. 4*a*, *b*). (It is assumed that the ascites cell with its very great powers of glycolysis will completely convert small amounts of glucose to lactate.)

Na transport persists in the absence of added substrate, owing either to continuing respiration or to the action of energy-rich phosphate bonds or to both, though k_2 (the rate coefficient for Na efflux), is less than in the presence of any other substrate, while k_1 (the rate coefficient for Na influx), is about the same. Calculation shows that in the earlier stages of incubation (0–10 min) Na efflux ($k_2.[\mathrm{Na_i}]_{\mathrm{mean}}$) is less in the absence of substrate, but at equilibrium (30–60 min) both effluxes are similar. This follows because at equilibrium efflux = influx, i.e. $k_2.[\mathrm{Na_i}]_\infty = k_1.[\mathrm{Na_e}]$. Hence if two different suspensions with the same external Na concentrations have the same values for k_1, the two influxes and hence the two effluxes must all be equal. Addition of cyanide to substrate-free suspensions results in decrease in k_2 and little change in k_1 (Table 1, no. 4).

Inosine. This substance raises respiration 20–30% above that found with added glucose, though if suspensions containing inosine + glucose and glucose alone are compared, respiration in the former is reduced nearly to the level of the latter (Table 1, nos. 1*b*–*d*; 2*c*, *d*; 3*b*, *d*; 6*a*, *b*). Aerobic glycolysis is comparatively slight, corresponding to 7–17 μmole glucose glycolysed/ml. cells.hr. On adding cyanide about 80% of the glucose presumed saved from respiration is turned over to glycolysis (no. 4*c*, *d*). k_2 in the presence of inosine is intermediate between that of substrate-free suspensions and suspensions containing glucose; k_1 is little affected. If glucose and inosine are present together in a suspension, glycolysis is slightly greater than when glucose is present alone, respiration is hardly affected, while k_2 is about the same or perhaps slightly greater.

Succinate. In the absence of glucose little or no accumulation of lactate occurs in suspensions with succinate added as substrate, but respiration is 10–20% higher than in suspensions containing glucose but no succinate (Table 1, nos. 2*b*, *c*; 7*a*–*c*); the addition of glucose to 'succinate' suspensions depresses respiration, though not quite to the level seen with plain 'glucose' suspensions. k_2 was rather less in simple 'succinate' suspensions than in 'glucose' suspensions, while k_1 was about the same in both. It may be noted that the addition of succinate to suspensions containing glucose did not raise k_2, and it was only used as a routine because its presence was essential when investigating suspensions poisoned with iodoacetate.

Galactose. With galactose as substrate glycolysis is about a quarter of that found with glucose, while respiration is about 50% greater; addition of glucose to 'galactose' suspensions increases glycolysis and lowers respiration to the level found in simple 'glucose' suspensions (Table 1, nos. 3*c*, *d*; 5*a–c*). Galactose seems to energize Na transport more effectively than inosine, but less well than glucose.

Table 1. Effects of various substrates on Na transfer, respiration and aerobic glycolysis of ascites cells depleted of natural substrate by washing and incubation. External medium: dialysed albumin 3% in NaCl solution (0·14 M) with added phosphate. $[Na_e]$ 140–150 m-equiv/l.; $[K_e]$ 8 m-equiv/l. External phosphate 7 mM. Temp. 35° C

No.	Substrate added (m-mole/l. medium)	$[Na_i]$ (m-equiv/l. cell water) at		k_2 (hr^{-1})	k_1 (hr^{-1})	Glucose (μmole/ml. cells.hr) assumed converted to	
		0 min	∞ min			CO_2	Lactate
1*a*	None	136	83	5·0	2·8	7·4	0
b	Inosine 5	127	64	6·3	2·8	10·0	17
c	Glucose 28	113	48	9·0	3·1	8·1	122
d	Glucose 28 + inosine 5	112	56	9·8	3·8	8·7	140
2*a*	None	117	60	6·0	2·5	7·3	0
b	Succinate 12·5	111	46	7·8	2·5	9·9	0
c	Glucose 28	111	45	8·7	2·7	7·4	122
d	Glucose 28 + inosine 5	117	45	8·9	2·7	7·7	134
3*a*	None	85	45	5·5	1·6	8·0	0
b	Inosine 10	73	35	6·7	1·6	9·8	13
c	Galactose 28	73	35	8·0	2·0	10·7	22
d	Glucose 28	73	33	8·8	2·0	6·6	82
4*a*	None	116	69	4·3	2·0	9·6	1·1
b	None + cyanide 2	124	92	2·9	1·8	3·1	4·3
c	Inosine 5	110	46	6·2	1·9	14·8	7·2
d	Inosine 5 + cyanide 2	120	85	3·4	1·9	3·8	16
5*a*	Galactose 28	—	—	—	—	12·2	—
b	Glucose 28	—	—	—	—	8·2	—
c	Glucose 28 + galactose 28	—	—	—	—	8·0	—
6*a*	Inosine 10	—	—	—	—	10·3	12
b	Glucose 28	—	—	—	—	7·7	120
c	Glucose 28 + inosine 10	—	—	—	—	7·7	135
7*a*	Succinate 12·5	—	—	—	—	8·8	—
b	Glucose 28	—	—	—	—	6·4	—
c	Glucose 28 + succinate 12·5	—	—	—	—	7·1	—
8*a*	Pyruvate 25	—	—	—	—	9·6	—
b	Lactate 25	—	—	—	—	9·8	—
c	Glucose 12·5	—	—	—	—	6·4	—

Glutamate and oxoglutarate. These substrates seem to reinforce glucose in the cases of certain tissues. Thus glutamate is necessary for adequate cation transport by brain (Terner, Eggleston & Krebs, 1950), and oxoglutarate for transport by guinea-pig kidney (Krebs, Eggleston & Terner, 1951). In the case of ascites cells the following were compared: (1) glucose + succinate, (2) glucose + succinate + glutamate (5 mM), (3) glucose + succinate + oxoglutarate (5 mM); the respective values for k_2 were 10, 9·4 and 10·2/hr; for k_1 5·9, 5·6 and 6·2/hr; for glucose respired 7·9, 8·1 and 7·6 μmole; for glucose glycolysed 125, 127 and 119 μmole/ml. cells.hr. Thus there seemed no advantage in the routine addition of glutamate or oxoglutarate to nutrient media for ascites cells.

Relation of glycolysis and respiration. It has been seen that substances which liberate lactate comparatively slowly, like galactose and inosine, are associated with a respiration rate which is higher than that of glucose, and the same is true of succinate which is respired without the simultaneous occurrence of aerobic glycolysis. As soon as glycolysis is increased by adding glucose to any of these substrates, respiration is depressed.

Action of deoxycortone acetate on Na transfer

When this substance was added to suspensions containing glucose in amounts between 1·25 and 12·5 μg/ml. no changes were seen in respiration, glycolysis, k_2 or k_1. With the agent at 125 μg/ml. suspension, k_2 fell slightly (from 12·9 to 11·2 hr), while respiration was depressed by 20% and glucose glycolysed increased by 30% (from 140 to 184 μmole/ml. cells.hr); it was thought that the latter results might be due to a non-specific depression of respiration by very high concentrations of deoxycortone acetate.

Action of poisons on Na transfer

Effects of cyanide. In these experiments cyanide was present in the preliminary incubation (depletion stage), in the cold stage and during the incubation proper. Table 2 and Fig. 1 show that with 0·5 m-mole NaCN/l. cell suspension, respiration is depressed by 70%, glycolysis increased by about 100%, while k_2 and k_1 are practically unaffected, the former being sometimes slightly increased. It may be thought that although records show inhibition of respiration over a 1 hr period, respiration during the first quarter hour, when fall of [Na_1] is most active, might well be normal. In fact, observations were always made for each quarter hour period, and inhibition found to be marked from the first. Thus in eight experiments manometric readings averaged 9·6 divisions in unpoisoned and 2·5 divisions in poisoned suspensions, while during the second quarter hour the figures were 9·3 and 2·2 divisions. Obviously the error in the cyanide figures is considerable, but there can be no doubt that inhibition is marked from the first. It may also be suggested that Na transport is maintained in the presence of cyanide because of a failure adequately to exhaust the reservoirs of energy-rich compounds by preliminary depletion. That this is not so is clear from Expts. 2 and 3 of Table 2, where parallel observations on suspensions containing iodoacetate show a definite decrease in k_2. Higher concentrations of cyanide ($>$2m-equiv/l.) lowered k_2, in part at least because inhibition of respiration was more marked, while glycolysis was not further increased (nos. 1 and 3); in part perhaps through some non-specific action on metabolism.

That respiration plays some part in Na transport by ascites cells, and that the maintenance of transport in the presence of cyanide in spite of decreased respiration is in fact due to increased glycolysis, may be shown by adding iodoacetate to 'cyanide' suspensions as in Table 2, no. 4*c*; here glycolysis is greater than in the normal control (4*a*), but respiration is reduced and k_2 falls.

TABLE 2. Effects of cyanide and iodoacetate on Na transfer, respiration and aerobic glycolysis of ascites cells depleted of metabolic reserves by preliminary washing and incubation. Cells were finally incubated in a medium containing 8 mM-KCl, 1·75 mM-$CaCl_2$, 1 mM-$MgCl_2$, 120–130 mM-NaCl, 12·5 mM-Na succinate, 25 mM glucose, with albumin 3 g and phenol red 0·00175 g/100 ml. Temp. 35° C

	Poisons added (m-mole/l. cell suspension)				Na fluxes at $t=\infty$ (m-equiv/l. cell water.hr)*	Glucose (μmole/ml. cells.hr) assumed converted to	
No.	NaCN	Iodoacetate	k_2 (hr^{-1})	k_1 (hr^{-1})		CO_2	Lactate
1*a*	0	0	8·4	3·8	550	10·4	55
b	0·5	0	8·7	3·2	460	2·3	155
c	2	0	7·0	3·0	440	1·9	135
2*a*	0	0	8·5	2·2	340	9·1	82
b	0·5	0	8·6	2·5	380	2·8	182
c	0	0·045	5·4	3·1	470	8·7	28
3*a*	0	0	11·0	3·6	530	8·3	68
b	0·5	0	10·1	3·1	460	1·5	128
c	2	0	9·3	3·1	460	1·0	134
d	0	0·035	6·7	2·9	430	8·6	35
4*a*	0	0	7·5	3·4	480	9·5	83
b	0·5	0	8·3	3·6	510	2·9	154
c	0·5	0·02	6·5	4·2	590	2·6	105
d	0·5	0·03	5·4	4·3	610	2·7	38
5*a*	0	0	10·0	4·5	710	10·2	68
b	0	0·035	6·7	3·8	600	10·0	37
c	0	0·07	5·2	3·4	530	9·1	20
6*a*	0	0	12·7	2·2	310	15·9	95
b	0	0·035	9·0	1·7	240	16·4	73
c	0	0·07	5·9	1·3	180	15·6	57
7*a*	0	0	9·1	2·4	350	9·8	108
b	0	0·02	6·2	1·7	240	10·3	50
c	0	0·05	4·2	1·5	220	10·6	10
8*a*	0	0	8·3	2·6	380	9·5	135
b	0	0·035	4·3	2·3	330	9·1	77
c	0	0·07	3·8	3·0	430	8·8	44
9*a*	0	0·025	10·5	4·9	750	9·1	80
b	0·5	0·025	7·4	4·8	740	2·5	87
c	0	0·035	5·8	3·9	600	8·6	31
d	0·5	0·035	5·3	4·4	670	2·6	43

* The fluxes at $t=\infty$ are equal and are calculated from $k_2 . [Na_i]_\infty$. To convert the fluxes from m-equiv/l. cell water.hr to pmole/cm^2.sec multiply by the approximate factor 0·07.

This may also be shown in another way: cyanide is added to glucose-free suspensions, reducing respiration by 68 %, but inducing very little glycolysis; in one such experiment k_2 was 8·8 in the standard glucose-containing control, 4·8 in the unpoisoned glucose-free suspension and 3·6/hr in the glucose-free suspension poisoned with cyanide, while k_1 values were 2·7, 2·5 and 3·4/hr. It may be noted that the addition of cyanide to the glucose-free suspension increased $[Na_i]_\infty$ from 80 to 130 m-equiv/l. cell water, suggesting that the substrate-free cells were seriously affected by the poison. The findings indicate that when glucose-containing suspensions are poisoned by cyanide the loss of energy from decrease in respiration is offset by gain from increased glycolysis, and so Na transport is maintained.

Malonate. This in a concentration of 12·5 m-mole/l. medium depressed respiration by 35% and increased glycolysis by only 5%; this doubtless explains the decrease in k_2 which was 5·1/hr compared with 6·5/hr in the unpoisoned control.

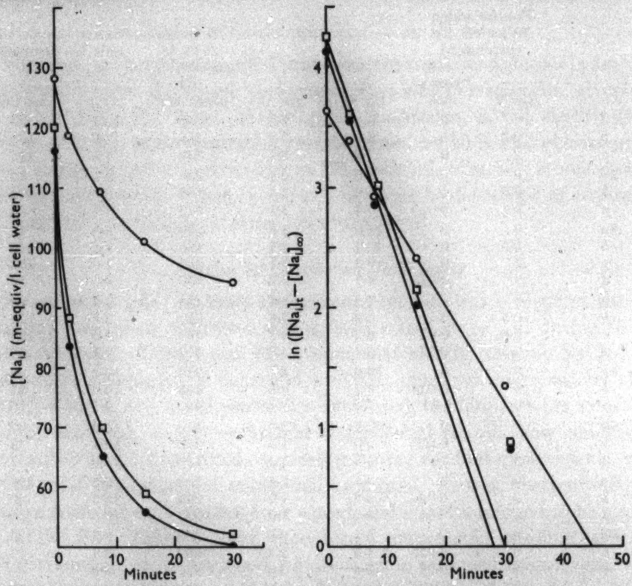

Fig. 1. Effects of cyanide (0·5 mM/l. medium), ●——●, and iodoacetate (0·035 mM/l. medium), ○——○, on the fall of [Na$_i$] in Na-rich cells at 35° C. Control, □——□.

Fluoroacetate. The sample used was unsatisfactory because when present in a concentration of 10 m-mole/l. it reduced glycolysis by 12%, presumably owing to a fluoride impurity. Respiration fell by only 10%, while k_2 was 5·1 compared with 6·2/hr in the control. The findings suggest that ascites cells are deficient in the specific activator, which, according to Buffa & Peters (1949), fluoroacetate requires in order to inhibit respiration.

L-*Triiodothyronine.* It is known that oxidative phosphorylation is uncoupled by thyroxine, though Tapley & Cooper (1956) believed this to be an effect secondary to change induced in the mitochondria. Triiodothyronine (0·1 m-mole/l. suspension) had very little effect on ascites cells, k_2 being 7·1 compared with 7·5/hr in the control; glucose presumed converted to CO_2 was 9·0 compared with 8·4 μmole/ml. cells.hr, and glucose glycolysed was 80 compared with 76 μmole/ml. cells.hr.

Iodoacetate (Table 2 and Fig. 1). This investigation is complicated by the fact that with the concentration of iodoacetate constant throughout an experiment glycolysis decreased progressively, and it may be presumed that if transport is affected, cell sodium concentration will not fall to a fixed low level; and in fact some of the curves obtained with iodoacetate are U-shaped, the $[Na_i]$ of the Na-charged cells falling, on incubation, to a minimum which is higher than that of the unpoisoned controls and then rising again. With lower concentrations of iodoacetate the late rise may be delayed beyond the usual period of observation, and the flat part of the curve then gives an apparent value for $[Na_i]_\infty$ which is spurious and high, and will give values for k_2 which are also false and high. To minimize this cumulative effect of iodoacetate the poison was only added towards the end of the preliminary 'depletion stage', and was absent from the medium used during the actual preparation of the time curve (see Methods).

Iodoacetate inhibits aerobic glycolysis in ascites cells and in larger amounts inhibits respiration too, presumably by a less specific effect on the SH-groups of the respiratory enzymes, the margin between concentrations causing simple inhibition of glycolysis and those showing commencing inhibition of respiration as well being comparatively small (e.g. 0·05 and 0·1 m-mole/l. suspension). Smaller amounts of iodoacetate, while causing some inhibition of glycolysis, left respiration unaffected, or were even associated with a slight increase in respiration (Table 2, nos. 3*a*, *d*; 7*a*, *b*), the findings then comparing with those in glucose-free cell suspensions containing inosine, galactose or succinate, where a low rate of glycolysis was associated with increased respiration (Table 1).

It is obvious that iodoacetate decreases the total energy available in the cells, and in accordance with this k_2 is reduced (Table 2). k_1 decreased with moderate amounts of iodoacetate, but with higher concentrations it was variable, being sometimes greater and sometimes less than k_1 of the control. Table 2 shows that with moderate amounts of iodoacetate there usually occurs some decrease of both the fluxes at equilibrium (nos. 3*a*, *d*; 5*a*–*c*; 7*a*–*c*): this is an expression of the fact that while k_2 is low, $[Na_i]_\infty$ is only a little raised. With higher concentrations of the poison, though k_2 falls still more, $[Na_i]_\infty$ is so high that both influx and efflux are sometimes increased (nos. 2*a*, *c*; 8*a*, *c*).

An attempt was made to increase glycolysis in suspensions poisoned with low concentrations of iodoacetate by adding cyanide, which in iodoacetate-free suspensions greatly increases lactate production. It was found, however, that in the presence of iodoacetate, cyanide had rather little effect (Table 2, no. 9), and k_2 so far from increasing, actually fell. This suggests that marked inhibition of respiration by cyanide was not compensated by the small rise in glycolysis, and also that both glycolysis and respiration contribute to Na transport by ascites cells.

The correlation of glycolysis with k_2 and with efflux itself is not good: this perhaps is not surprising when it is recalled that efflux depends not only on a coincident metabolic cycle, but also on the survival and mediation of substances with energy-rich phosphate bonds.

DISCUSSION

It has long been known that while normal tissue cells respire freely in the presence of oxygen and produce very little lactate, tumour cells respire less actively and produce much lactate even in the presence of oxygen, glycolysis being further increased when respiration is suppressed (for Warburg's early work and other references, see Warburg, 1956); this is true also of ascites cells (Warburg & Hiepler, 1952). In the present work, with ascites cells not in their natural medium but in an albumin–succinate–glucose medium, respiration has varied widely between 1000 and 2000 μl. O_2 used/ml. cells.hr, and lactate formed has varied between 100 and 300 μmole/ml. cells.hr: assuming that both CO_2 and lactate formed are derived from glucose, the figures correspond to 7–15 μmole respired and 50–150 μmole glycolysed/ml. cells.hr. In the absence of K respiration is slightly and glycolysis greatly decreased (Maizels *et al.* 1958*b*). When cells are depleted of natural substrate by washing and preliminary incubation, respiration continues for several hours, while glycolysis is not perceptible. On adding cyanide to such a preparation glucose saved from respiration is about twice as great as that diverted to glycolysis, indicating that the CO_2 of endogenous respiration is only partly derived from glucose as such. Endogenous respiration in normal and ascites cells has recently been investigated by Kalant & Young (1957).

Ascites cells manifest a well-marked Pasteur effect: Warburg (1956) gives figures showing 100% increase in glycolysis; Table 2 shows increases up to 200%. In the present investigation it was also found that ascites cells showed a sort of 'Pasteur effect in reverse'; thus if for any reason glycolysis is decreased, respiration rises. Such a decrease may arise from paucity of natural substrate, or because an abnormal substrate is utilized so slowly that the actual yield of lactate is small. Thus well-washed ascites cells with no added substrate respire as well as, or better than, glucose-containing suspensions for periods of 2 hr or more (Table 1), while the addition to washed cells of inosine or galactose which show poor yields of lactate gives figures for respiration which are 30–50% higher than those of suspensions containing glucose; the same is true of succinate, which is respired directly without the formation of lactate. But the addition of glucose to suspensions containing inosine, succinate or galactose reduces respiration to the levels found with glucose alone. The findings suggest that glycolysis and respiration share a common co-factor, which ordinarily is monopolized by the highly active glycolytic system.

However, if the co-factor were diphosphopyridine nucleotide—and this is a possibility—the release of respiration when glycolysis is inhibited becomes less easy to explain, for in the case of normal tissues at least that part of cell DPN devoted to respiration is 'built into' the mitochondria (Lindberg & Ernster, 1954), and it is unlikely to be available as a co-factor in glycolysis. On the other hand, Kielley (1952) and Williams-Ashman & Kennedy (1952) have shown that the addition of DPN is necessary in order to obtain high rates of oxidation of respiratory intermediates by tumour cell mitochondria, and it is possible that DPN in solution in the cell and ordinarily devoted to sustaining the very rapid glycolysis of the ascites cell might be diverted to the use of the mitochondrial system when glycolysis is depressed. Naturally, this phenomenon does not occur with normal cells, where glycolysis is so adjusted to respiration that no accumulation of lactic acid occurs.

Inosine. In the case of erythrocytes, nucleosides are split by nucleoside phosphorylase, the purine possibly being excluded, while the ribose simultaneously combined with inorganic phosphate is incorporated with the cell, where it contributes to the sum of energy by the ultimate conversion of ribose phosphate to lactate. The phosphate portion is also available for the rephosphorylation of AMP and ADP where, as in the case with cold-stored erythrocytes, the reserves of adenosine triphosphate are low (Prankerd & Altman, 1954; Gabrio, Donohue & Finch, 1955). Hence adenosine, with or without glucose, has little effect on fresh erythrocytes which are rich in ATP, but raises glycolysis and Na transport in stored erythrocytes to normal levels (Clarkson, 1956). In the case of washed ('depleted') ascites cells with added inosine and inorganic phosphate, respiration is much more than in the presence of glucose, while more lactate is formed than in suspensions without added substrate, the extra lactate presumably being derived from ribose. In accordance with this, k_2 is greater than in suspensions without added substrate, though less than k_2 in suspensions containing glucose. If glucose is already present, the addition of inosine as well causes only slight further increase in glycolysis, and it is doubtful if there is any significant increase in k_2 (Table 1, no. 2*c*, *d*): hence either inosine fails to increase ATP in the cells, or else ATP is not a limiting factor in transport, even in the cell depleted by washing and preliminary incubation.

Galactose. In many tissues this is less easily metabolized than glucose: this is so with sarcoma slices and brain homogenates (Meyerhof & Geliazkowa, 1947), and also in the case of human erythrocytes which utilize very little galactose (Maizels, 1951; Schwarz, Golberg, Komrower & Holzel, 1956); recently we have found by direct measurement of lactate produced that human erythrocytes glycolyse glucose 6–8 times as fast as galactose. With ascites cells lactate formation from galactose is about one-quarter of that found with glucose, while respiration is about 50% greater; presumably because of the latter, k_2 is only slightly less than in the presence of glucose, and is greater than in the inosine preparations (Table 1, no. 3).

Glycolysis, respiration and Na transport. It has been seen that both glycolysis and respiration contribute to Na transport by ascites cells, but owing to all three showing individual variations, and because efflux includes an unmeasured fraction based on reserves of energy-rich phosphate bonds, it is impracticable to gauge the individual contributions of energy to transport. It is, however, possible to say in experiments like 1*a* and *b* (Table 2), where k_2 is roughly constant, that the loss, induced by cyanide, of 8·1 μmole glucose respired requires that in its stead 100 μmole glucose be glycolysed if efflux is to be maintained. In eight experiments where in the presence of cyanide k_2 remained roughly constant, the ratio of respiration inhibited to increase of glycolysis expressed in terms of glucose was 1:15 (range 1:10–1:18). According to Krebs & Kornberg (1957) the ratio of energy-rich phosphate bonds yielded respectively by glycolysis and respiration is 2:36; clearly the two ratios are of the same order, and this suggests that, apart from the extravagance inherent in using glycolysis rather than respiration, there is little additional loss of efficiency when the ascites cell turns from respiration and glycolysis to glycolysis alone. The data also suggest that in the unpoisoned cell (where the ratio of glucose glycolysed to glucose respired averages about 10·5:1) the energy contribution of respiration is over one and a half times that of glycolysis.

These findings may be compared to those of other cells. Active cation transport by kidney slices is inhibited by anoxia and 2:4-dinitrophenol (see Davies, 1954). Retina normally manifests respiration and aerobic glycolysis, respiration continuing undiminished at very low oxygen tensions, while glycolysis is much increased (Laser, 1937); under anaerobic conditions cation transport is reduced but not abolished (Terner *et al.* 1950). In human erythrocytes transport is based on glycolysis and not on respiration (Harris, 1941; Maizels, 1951), while in tortoise erythrocytes transport is normally based on respiration, but the inhibition of respiration by cyanide, with or without the simultaneous inhibition of glycolysis by iodoacetate, does not decrease Na efflux, at least for an hour or two (Maizels, 1956); frog muscle shows a similar resistance (Keynes & Maisel, 1954). In either tissue so poisoned the transport is probably based on energy-rich phosphate bonds; however, the observations do not exclude the possibility that glycolysis when it occurs may contribute to transport. Normally, chicken erythrocytes also respire without producing lactate, and Clarkson & Maizels (1955) showed that if cyanide were added to washed chicken and human erythrocytes then for the same amount of glycolysis the naturally glycolysing human cell transports Na more efficiently than the chicken erythrocyte in which normal respiration has been suppressed. They considered that the residual active transport seen in their washed and stored chicken erythrocytes was based on energy-rich phosphate bonds. Tosteson & Robertson (1956), using *fresh* duck erythrocytes, found K influx to be greater in nitrogen than under aerobic conditions. The difficulty in rendering haemoglobin-rich materials completely O_2-free is considerable, and it is possible that both glycolysis and respiration were occurring together at very low oxygen tension (cf. Laser, 1937, above). There are thus tissues which can only transport cations by means of glycolysis (mammalian erythrocytes), or only by means of respiration; others like nucleated erythrocytes which normally base transport on respiration, but can transport with energy supplied by glycolysis, and still others like retina and embryonic tissues which manifest respiration and aerobic glycolysis simultaneously. Ascites tumour cells belong to the latter group, though they respire less and produce far more lactate than any of the others (Warburg, 1956), and in the presence of cyanide they are able to increase glycolysis still further, and so maintain cation transport, in spite of failure of respiration.

Concerning energy requirements for a coupled Na-K pump: at equilibrium no electrical work is done, and so no figure is required for the membrane potential, though this might perhaps have been obtained from the chloride concentration ratio. Work done may then be calculated from the equation of Keynes & Maisel (1954) rewritten as follows:

$$\text{work done} = k_2.[\mathrm{Na_i}]\,F(E_{\mathrm{Na}}+E_{\mathrm{K}})$$

where F is taken as 23,100 cal/V.mole,

$$E_{\mathrm{Na}}=\frac{RT}{F}\ln\frac{[\mathrm{Na_e}]}{[\mathrm{Na_i}]_\infty}\quad\text{and}\quad E_{\mathrm{K}}=\frac{RT}{F}\ln\frac{[\mathrm{K_i}]_\infty}{[\mathrm{K_e}]}.$$

In Expt. 2 of Table 2, $k_2=8{\cdot}5$/hr, $[\mathrm{Na_e}]=156$ and $[\mathrm{K_e}]=8$ m-equiv/l. water in the external medium, while $[\mathrm{Na_i}]_\infty=40$ and $[\mathrm{K_i}]_\infty=144$ m-equiv/l. cell water. Hence energy required is 1150 cal/l. cell water.hr. Assuming that each energy-rich phosphate bond is worth 13,500 cal, energy produced by this ascites cell is 6600 cal/l. cells.hr or 7700 cal/l. cell water.hr; this does not include energy available from pre-existing ATP. Thus energy required for cation transport is something under 15% of that available.

SUMMARY

1. If ascites cells are depleted of natural substrate by washing and incubation, respiration sometimes continues unreduced for some hours, only part of the CO_2 produced being derived from residual carbohydrate.

2. In the presence of added substrates, which are associated with little or no glycolysis (inosine, galactose, succinate), respiration is 30–50% greater than with added glucose. The addition of glucose along with inosine, galactose or succinate depresses respiration to the level seen with glucose alone.

3. The marked depression of respiration induced by small amounts of cyanide is offset by a marked increase in glycolysis, and no decrease in active Na transport occurs.

4. Iodoacetate in small amounts greatly depresses glycolysis leaving respiration unaffected. Available energy decreases and cation transport in 'depleted' cells fails. The addition of cyanide to cell suspensions poisoned with iodoacetate still further depresses active transport, showing that respiration as well as glycolysis is concerned in transport.

5. Energy available to the ascites cells from respiration exceeded that available from glycolysis and might be twice as great. Of this only 10–20% was required for active transport.

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THE ABSORPTION OF CHLORIDE IONS FROM THE RETICULO-RUMEN SAC

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Chloride ion can move against its concentration gradient to the blood from the contents of the reticulo-rumen of sheep (Parthasarathy, 1952; Parthasarathy & Phillipson, 1953; Sperber, Hydén & Eckman, 1953) and from the rumen pouch of the goat (Sperber & Hydén, 1952). The object of the work presented here is to explain this transport in terms of the forces moving the ion.

An ion which diffuses freely through a membrane should move down its electrochemical gradient. The electrochemical potential difference in volts of an ion between two solutions denoted by subscripts 1 and 2, $(\mu_1-\mu_2)/F$, is given by the following expression

$$\frac{\mu_1-\mu_2}{F}=\frac{RT}{F}\ln\frac{c_1}{c_2}+\frac{RT}{F}\ln\frac{f_1}{f_2}+z(V_1-V_2),$$

where c is the concentration of the ion, f is its activity coefficient, z the ion charge and V the potential. The separate evaluation of the potential difference and activity coefficient cannot be justified thermodynamically, as measurements can be made which lead to only certain combinations of these two quantities (e.g. Harned & Owen, 1950). Nevertheless, the measurement of the potential between two aqueous phases by strong KCl bridges making contact with reversible electrodes, with correction, if necessary, for differences in the activity coefficients, has given results of considerable significance in studies of permeability, particularly of frog's skin and of nerve fibres. The potential difference between the blood and the contents of the reticulo-rumen sac of unanaesthetized sheep with normal rumen contents was found to be of a size and direction which would account for the movements of chloride against its concentration gradient. Acute experiments were then undertaken to discover whether it would account for the direction of chloride movement in detail. A preliminary account of these experiments has been given (Dobson & Phillipson, 1954).

METHODS

Electrical measurements on conscious sheep

Sheep were fitted with a permanent ebonite rumen cannula at least a month before the experiment. Measurements were made with the animal lightly restrained on a low wooden stand. The potential differences were measured every 1 or 2 min to ±0·5 mV, using a Cambridge pH meter with floating earth. Saturated calomel electrodes were used, dipping into saturated KCl at room temperature in a water-bath. Saturated KCl–agar bridges led from the saturated KCl to the animal. These bridges were made by filling polythene tubing with hot solution containing 40% KCl and 3% agar. A bridge in tubing of 0·5 mm bore was used to make contact with the blood by inserting it through a wide bore hypodermic needle into the jugular vein. Contact to the rumen contents was made by two bridges in polythene tubing of 2 mm bore. These were inserted into the rumen contents through glass tubes in a bung in the rumen cannula. The tip of each bridge was protected by a polythene sheath at the end of each glass tube.

Acute experiments

Surgery. The surgery for these experiments has been described in detail (Masson & Phillipson, 1951). The rumen of a small sheep was cannulated at least a month previously. After fasting during the night, the reticulo-rumen sac contents were washed out and replaced with sufficient 150 mM-NaCl. The sheep was then anaesthetized with pentobarbital and the sac isolated from the rest of the gut by a ligature round the omasal neck, and another round the oesophagus. Before the abdomen was closed a small bridge was introduced into a small rumen vein draining the right side of the posterior part of the ventral sac, and passed into the main posterior rumen vein.

Absorption experiment. The saline in the sac was washed out with tap water at 39° C to reduce to a minimum the chloride remaining. The experimental solution was then introduced and, after approximately half an hour, was removed and the rumen drained. The residue of the experimental solution was washed out by introducing immediately 3 l. of warm tap water which was then removed, and the rumen drained. As soon as possible the next experimental solution was poured in. The volume of solutions and water introduced into and removed from the rumen was measured in cylinders with a S.E. of ±2·5 ml. and corrected to 39° C.

To allow corrections to be made for the residues left in the rumen, the emptying of the rumen and the washing were carried out in a standard way, and all operations were timed. The residue of the experimental solution, about 50 ml., was calculated by determining the acetate content of the tap water with which the rumen was washed. Acetate was chosen as a marker because it was found that no detectable quantity of this ion entered a volume of tap water introduced for a short time into an empty rumen previously washed out with tap water. This was not true for sodium, potassium and chloride. The residues of the wash solutions were assumed to be the same as the residues from the experimental solutions, as these were drained in the same manner. Loss of water from tap water residues would be greater than from the experimental solution residues during the short time between draining the rumen and introducing the next solution, as the experimental solutions were approximately isotonic with plasma. It has been assumed that the loss of water while the wash solution was in the rumen, approximately 40 ml. in 3 min, continued linearly up to the time the experimental solution was introduced. This assumption permitted a calculation to be made of the volume of residue at the instant the experimental solution was introduced into the sac, thus allowing the initial volume for an absorption period to be estimated. The error in calculating the water changes during an experimental period was probably not greater than ±20 ml.

Chloride entered the wash solution to the extent of 0·5 m-equiv in 3 min. This entry was assumed to continue linearly until the experimental solution was introduced. This small correction for entry plus the amount of chloride left behind in the wash residue were added to the amount introduced in the experimental solution. When the net chloride movement was small the application of these small corrections to the change of chloride in the rumen allowed more confidence to be placed on the results. Leakage of chloride into the rumen from the bridges was found to be negligible.

It was found that the errors in chloride flux measurement lay mainly in the method of analysis and in measurement of the volumes of experimental solution introduced and recovered from the rumen. As fractional changes in volume and concentration during an absorption period were small, the errors were estimated from the expression

$$d\Delta = \sqrt{[2(V^2\, dc^2 + c^2\, dV^2)]},$$

where $d\Delta$ is the S.E. of the change in chloride; V is the mean volume, with S.E. dV; c is the concentration, with S.E. dc.

The electrochemical potential difference was calculated from the following values:

(1) The concentration contribution was calculated from the mean of the calculated initial concentration and the measured final concentration in the rumen, together with the plasma chloride from a sample from the jugular vein taken halfway through the absorption period. As it was not practical to collect plasma samples anaerobically, 4% was subtracted from the measured concentration to allow for chloride shift on exposure to air.

(2) The activity coefficient ratio was assumed to be unity, as the tonicity, and presumably the ionic strength of the solutions used, was close to that of the plasma.

(3) The potential between two bridges in the rumen contents and a bridge in the venous blood leaving the rumen was measured. When the reading of the two electrodes differed, the two readings were averaged. The mean value over the absorption period was taken as the potential contribution to the electro-chemical potential difference. To obtain steady readings it was necessary to keep the sac contents stirred by pressing rhythmically on the sheep's abdomen.

Analysis. Chloride was determined by electrometric titration with $AgNO_3$ in 50% (w/v) acetic acid (Sanderson, 1952). Analysis of experimental solutions was made using a differential technique (Keys, 1931) which gave a standard error of duplicate estimations of ±0·012 mM for concentrations up to 50 mM. Protein interference was negligible. Plasma chloride was found by titrating suitably diluted samples (S.E. <1%). The wash solutions were titrated directly with a similar accuracy. Acetate was determined as steam volatile fatty acid with a S.E. of ±0·6 mM. For 1·5 l. of 150 mM acetate, the total S.E. in acetate uptake is ±1·4 m-equiv.

Conventions. A net flux is given a positive sign if its direction is from rumen contents to plasma. The electrochemical potential difference and its components are measured with respect to the plasma. This means that the sign of the electrochemical potential will be the same as the sign of the flux for an ion which moves down its electrochemical potential difference.

RESULTS

Electrical potential difference across reticulo-rumen epithelium

Stability of potential reading. Handling the bridges or dipping a loop into water had no effect on the potential difference measured. If an end artifact was suspected the removal of the end centimetre of the bridge laid bare a fresh surface of KCl–agar with which to renew contact. This was done frequently at first, until it was clear that no appreciable change in potential resulted from this operation (Fig. 1). Touching the meter did not affect the readings except under very damp conditions in the sheep pens. This effect was eliminated by standing the meter and observer on polythene sheeting. The potentials measured showed no change, transient or otherwise, on moving the rumen bridge through the contents, or when the sheep bleated or fidgeted.

Potential differences within the reticulo-rumen contents. The potential difference was measured between a bridge with its tip fixed a few centimetres inside the rumen cannula and another with its tip at a distance from the cannula which could be varied. This potential difference was less than ± 0·5 mV from within the lumen of the ebonite cannula (in the dorsal sac of the rumen), and across the contents of dorsal and ventral sacs. Whenever the movable bridge tip touched the rumen wall it became positive, with a fluctuating potential difference of up to 14 mV.

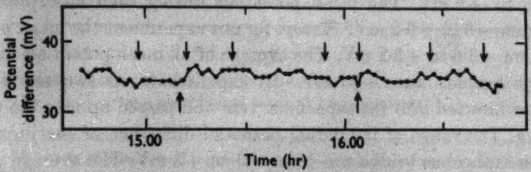

Fig. 1. Potential of jugular bridge with respect to rumen bridge. The arrows above the record indicate where the rumen bridge tip was cut off to renew the surface of KCl. The arrows below the record show where the jugular bridge tip was renewed.

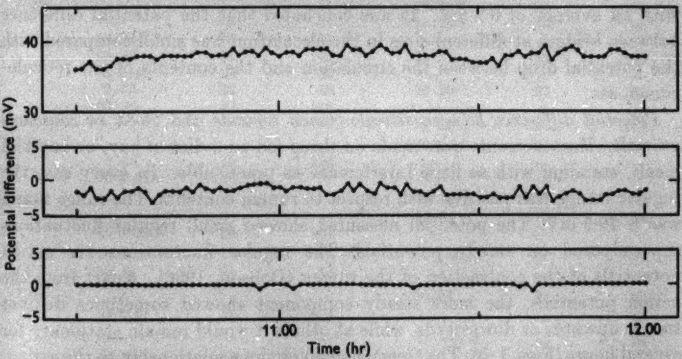

Fig. 2. Top record: potential of jugular bridge with respect to rumen bridge 1; middle record: potential of reticulum bridge with respect to rumen bridge 1; bottom record: potential of rumen bridge 2 with respect to rumen bridge 1. The reticulum bridge was inserted through a reticulum cannula. The lower records were obtained by difference which probably accounts for the small differences appearing between the two rumen electrodes.

The potential difference between the reticulum and rumen was measured in one sheep which had been successfully fitted with a polythene cannula in its reticulum. Similar results were obtained with other sheep when the reticulum bridge tied to a recording balloon was introduced by way of the rumen cannula. Small potential differences of from 0 to 4·5 mV with the rumen positive were measured (Fig. 2). It was concluded that potential gradients across the reticulo-rumen contents were small compared to the potentials between the contents and the blood.

Potential differences between different sites in the blood. In acute experiments when the potential between the blood leaving the rumen and rumen contents was measured, another connexion to the blood was made by inserting a bridge into the jugular vein. A small potential difference was often found between the bridges in the circulation. Individual readings of jugular with respect to rumen bridge during twenty-nine absorption periods on eight sheep ranged from −6 to +6 mV. The mean potentials during each absorption period ranged from −6 to +3·2 mV. Except for one experiment the mean potentials varied from −3·6 to +3·2 mV. The average of all mean potentials during the absorption periods was −1·1 mV. In experiments on two sheep a third bridge was inserted into the saphenous vein and passed up into the posterior vena cava. The range of individual potential difference of the jugular with respect to saphenous bridge was from −3 to +3 mV. The average potential difference during ten absorptions ranged from −1·9 to +1·6 mV, with an average of −0·5 mV. The calculated mean potential difference of the saphenous bridge with respect to the rumen bridge ranged from −1·8 mV to +2·4 mV with an average of 0·2 mV. It was concluded that the potential difference between bridges at different sites in the circulation was small compared with the potential drop between the circulation and the contents of the reticulo-rumen sac.

Potential difference between reticulo-rumen contents and blood in conscious animals. Measurements were made on sheep fed on a diet of hay, or hay and meals, standing with as little interference as practicable. In every case the jugular bridge was positive with respect to rumen contents. The range found was 8–49·5 mV. The potential measured showed small regular fluctuations superimposed on steady potentials. The regular fluctuations are action potentials of the contraction of the rumen (Dobson, 1956). Apart from the action potentials, the more steady component showed sometimes distinct trends upwards or downwards, while at others it would remain stationary for several hours (Figs. 1–3). The trends had no obvious relationship to the eating, drinking or ruminating of the sheep. Some of the more steady records were obtained the first time the sheep was introduced into the stand, although the animal was nervous and difficult to pacify. The range of potential of each recording period is shown in Table 1, together with a representative selected potential. The latter represents the potential during the first period of 15 min or more in each recording when the potential remained fairly constant. Only one period was taken from each morning or afternoon recording to give potentials fairly independent of each other. The mean potential over the recording period was close to the potential chosen.

The mean of these selected results is 31·2 mV, S.D. ±6·8. The contribution to the electrochemical potential difference for a univalent ion of a potential difference of this size is equivalent to that of a three times concentration ratio.

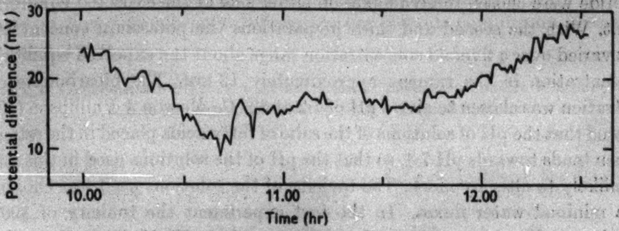

Fig. 3. Potential of jugular bridge with respect to rumen bridge. The line is drawn to join observations taken at 1 min intervals.

TABLE 1. Potentials of jugular vein with respect to rumen contents in conscious sheep. The basis of selection of representative potentials is discussed in the text

	Morning			Afternoon		
Sheep no.	Range (mV)	Selected (mV)	Recording time (min)	Range (mV)	Selected (mV)	Recording time (min)
1040	—	—	—	33–36·5	35	125
	—	—	—	21–38·5	34	66
	18–34	25	156	—	—	—
	25–35	34	167	19–27	25	176
	32·5–40	38	82	—	—	—
	9–25	22	80	25–30	27	61
	23–32	27	108	—	—	—
	—	—	—	22–28	26	140
1048	—	—	—	29·5–34	30	32
	9–28	16	152	—	—	—
	20·5–31	26	69	—	—	—
449	—	—	—	38–43	41	60
	—	—	—	34–38	35	26
	35·5–39·5	38	157	—	—	—
0	26–35	30	40	31·5–35	32	20
351	29–34	31	16	—	—	—
1050	—	—	—	30–34	32	60
32	29·5–31	30	24	—	—	—
	—	—	—	47·5–49·5	49	20
30	35–38	36	17	—	—	—
Mabel*	—	—	—	28·5–33	31	60

* Mabel was a red poll non-lactating heifer.

Thus the concentration of chloride ion in the reticulo-rumen sac at equilibrium should be about one-third of the plasma concentration if the ion moves by free diffusion through the epithelium. As this is near the level found in acute experiments by Parthasarathy & Phillipson (1953) it seemed promising to measure simultaneously the flux and electrochemical gradient of chloride during acute experiments.

Chloride flux measurements

Acute experiments were performed on three sheep, each with five experimental solutions placed in the isolated reticulo-rumen sac for about half an hour. The composition of the solutions is given in Table 2. Concentrations of chloride were chosen to give fluxes on either side of the expected equilibrium point. With the second and third preparations the potassium concentration was varied over a fivefold concentration range about the expected equilibrium concentration in the rumen—approximately 12 mM. The bicarbonate concentration was chosen to give a pH of 7·2 at 39° C. Masson & Phillipson (1951) showed that the pH of solutions of the salts of fatty acids placed in the reticulo-rumen tends towards pH 7·4, so that the pH of the solutions used in this study is unlikely to alter by much. The tonicity of the solutions used was chosen to give minimal water fluxes. In the first experiment the tonicity of 390 m-osmolar produced movements of water into the sac (Table 3), and so it was adjusted to 347 and 360 m-osmolar in the second and third preparations

TABLE 2. Composition of experimental solutions, made up in tap water and equilibrated with 95% O_2–5% CO_2 at 39° C

		Concentration of ions (mM)				
Expt.	Soln.	Na^+	K^+	Cl^-	Ac^-	HCO_3^-
34	1	174·7	15	15	166	8·7
	2	174·7	15	70	111	8·7
	3	174·7	15	25	156	8·7
	4	174·7	15	50	131	8·7
	5	174·7	15	40	141	8·7
36	1	158·7	15	35	130	8·7
	2	153·7	20	25	140	8·7
	3	148·7	25	40	125	8·7
	4	168·7	5	50	115	8·7
	5	163·7	10	15	150	8·7
38	1	160	20	20	151·3	8·7
	2	170	10	30	141·3	8·7
	3	170	10	20	158·3	1·7
	4	160	20	30	148·3	1·7
	5	175	5	15	156·3	8·7
Tap water	—	0·8	0·06	0·8	—	—

TABLE 3. Ion concentration changes, and amounts absorbed from solutions placed in the isolated reticulo-rumen sac

		Chloride concn. (mM)			Acetate concn. (mM)		Uptake from sac		
Expt.	Period	Initial	Final	Plasma*	Initial	Final	Water (ml.)	Chloride (m-mole)	Acetate (m-mole)
34	1	16·2	17·02	106	167	152	−30	−2·08	26
	2	69·4	65·67	105	110	101	−90	+1·59	8
	3	26·3	26·23	104	152	144	−65	−1·60	6
	4	50·2	48·70	103	130	120	−36	+1·17	14
	5	40·6	39·74	102	141	132	−45	−0·10	11
36	1	35·4	33·98	96	126	116	+ 5	+2·96	22
	2	25·0	24·72	94	137	129	− 5	+0·40	15
	3	39·5	37·75	96	123	118	+44	+5·21	15
	4	49·0	48·00	96	112	107	+18	+2·82	10
	5	15·3	15·49	96	144	138	−15	−0·54	8
38	1	20·2	22·78	97	144	125	+16	−3·88	32
	2	30·1	31·31	94	134	118	−10	−2·34	25
	3	20·4	21·69	93	150	133	− 1	−2·24	28
	4	30·0	30·01	93	141	125	−17	−0·56	24
	5	15·4	17·69	92	148	132	+ 4	−3·72	27

*Corrected for Cl^- shift.

respectively. The resulting reduction in water flux minimized the possibility of solvent flow through pores influencing the chloride equilibrium (Ussing & Andersen, 1956).

TABLE 4. Components of the electrochemical potential difference of chloride with respect to plasma and chloride fluxes with respect to rumen contents

Expt.	Period	E_{Cl}* (mV)	zV (mV)	E_{Cl}* + zV (mV)	Chloride flux (μequiv/min)	95% half confidence interval (±μequiv/min)
34	1	−49·6	44·0	− 5·6	− 63	4
	2	−11·8	28·1	16·3	48	15
	3	−36·3	31·0	− 5·3	− 48	6
	4	−19·7	32·3	12·6	35	11
	5	−25·1	36·0	10·9	− 3	9
36	1	−27·2	36·5	9·3	91	8
	2	−35·9	37·4	1·5	13	6
	3	−24·6	35·8	11·2	163	9
	4	−18·3	25·4	7·1	86	11
	5	−49·1	33·1	−16·0	− 17	4
38	1	−40·4	34·2	− 6·2	−121	5
	2	−30·2	26·6	− 3·6	− 74	7
	3	−39·9	30·2	− 9·7	− 70	5
	4	−30·4	28·3	− 2·1	− 18	7
	5	−46·1	30·5	−15·6	−118	4

*$E_{Cl} = -62 \log c_{Pl}/c_{sac}$.

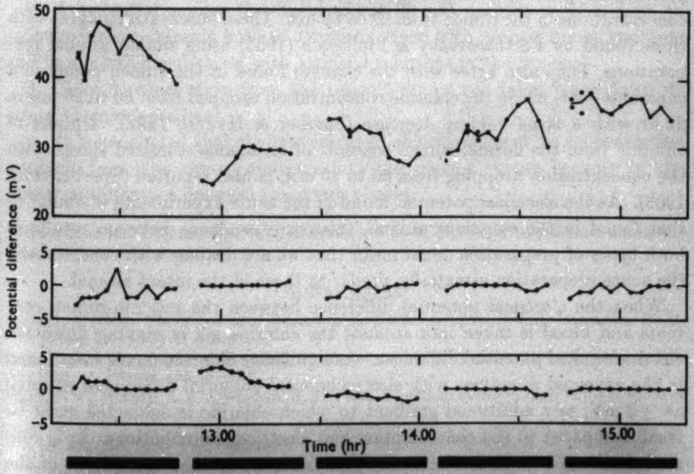

Fig. 4. Potential record of Expt. 34. Top record: potential of rumen artery bridge with respect to rumen bridges 1 and 2. Line joins mean of the two readings where they differ. Middle record: potential of jugular bridge with respect to rumen artery bridge. Bottom record: potential of jugular bridge with respect to saphenous vein bridge. The solid bars represent the length of the absorption periods.

The changes in concentration of chloride and acetate, and the changes in amount of water, chloride and acetate, for the three acute experiments are given in Table 3. The contributions of the electrical potential and concentration ratio to the electrochemical potential are given in Table 4. It is not possible to calculate an error to associate with the mean potentials. The potential record of the first acute experiment (Fig. 4) is given to show how the individual potential readings could vary during the course of an absorption period. In this record the rumen blood bridge was inserted into a rumen artery. In the other experiments a rumen vein was used.

DISCUSSION

The acute experiments on the sheep were designed to allow a comparison between the net chloride movement between the rumen contents and blood and the driving forces involved. One component of the driving force, the electrical potential, was within the range encountered in unanaesthetized sheep with normal rumen contents. Thus the range of mean potentials found for the fifteen absorption periods was 22–44 mV.

In one experimental period the flux of chloride failed to reach a value significantly different from zero (Expt. 34, period 5). Of the fourteen remaining periods, six gave movements against the chloride concentration ratio with concentrations in the rumen from 25 to 69 mM. These observations agree with those found by Parthasarathy & Phillipson (1953) using similar animal preparations. They also agree with the changes found in the rumen pouch in a conscious goat, where the chloride concentration dropped from 86 to 25 mM in 22 hr with a large volume decrease (Sperber & Hydén, 1952). Uptake of chloride from the normal rumen contents of an unanaesthetized sheep, with the concentration dropping from 60 to 25 mM, is also reported (Sperber *et al.* 1953). As the electrical potential found in the acute experiments is similar to that found in the conscious animal, this correspondence between results in both types of preparation could mean that we are dealing with conditions in the acute preparation essentially similar to those in the intact animal.

When the electrical potential difference between the reticulo-rumen contents and blood is taken into account the chloride ion is moving down the electrochemical potential difference. As significant chloride fluxes were found in the expected directions with electrochemical potential differences as small as ±2 mV, any additional gradient to which chloride is subjected must be small compared to the concentration and electrical contributions. It is concluded, therefore, that chloride diffuses freely across the epithelium of the reticulo-rumen sac. This statement requires some qualification, as the results obtained give no information on the possibility that the fluxes of chloride in both directions interact. To explore this possibility would naturally require the measurement of these component fluxes. In frog skin the component fluxes of chloride do not interact (Koefoed-Johnson, Levi & Ussing, 1952), but component fluxes of phosphate (Mitchell, 1953) and of potassium (Hodgkin & Keynes, 1955) are known to interact at cellular membranes.

It seems unprofitable to define any sort of permeability constant, in the sense of flux per unit driving force. For instance, in Expt. 36 the flux per unit driving force during the first four periods is 10, 9, 15 and 12 μequiv/min/mV, where as in the fifth period it drops to 1 μequiv/min/mV. This means that caution is necessary in comparing results of one experimental period with another for chloride in this kind of acute experiment.

In all experimental periods an uptake of acetate was found, consistent with previously published results (e.g. Masson & Phillipson, 1951).

The small potential differences between the reticulum and rumen contents may be due in part to the difference in composition of the contents of these organs (Gray, Pilgrim & Weller, 1954). The potentials between different sites in the blood are too large to be explained by differences of composition. Their origin remains obscure.

SUMMARY

1. Potential gradients within the reticulo-rumen contents and within the blood are small.

2. A potential makes the blood about 30 mV positive with respect to reticulo-rumen contents both in conscious sheep with normal rumen contents and in anaesthetized sheep with artificial solutions in the rumen.

3. Chloride absorption from the reticulo-rumen sac takes place down its electrochemical gradient.

4. It is concluded that the movement of chloride against its concentration gradient from the rumen contents into the plasma occurs because of the potential difference between these two phases.

We wish to thank Mr and Mrs W. C. B. Brown and Mr L. E. Vowles for assistance during the course of these experiments.

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THE RELATIONSHIP OF 5-HYDROXYTRYPTAMINE TO CAPILLARY PERMEABILITY IN THE SKIN OF THE RAT

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The characteristic regional pattern of blueing of the skin of guinea-pigs after intravenous injections of vital blue dyes and histamine liberators is directly related to the histamine content of the skin, and thus can be used as an indicator of histamine liberation in this species (Feldberg & Miles, 1953). The blueing, which is antagonized by mepyramine, is apparently due to an increased capillary permeability following the release of histamine. It is not known whether a similar relationship exists in rats between the histamine content of different skin areas and their readiness to develop oedema.

The skin of the guinea-pig contains histamine but very little 5-hydroxytryptamine (5-HT), whereas that of the rat contains much of both amines (Parratt & West, 1957*a*). Recently, Rowley & Benditt (1956) have shown that 5-HT is nearly 200 times as active as histamine on subcutaneous injection in increasing capillary permeability in the rat, and Sparrow & Wilhelm (1957) report it to be more than 11 times as active on intracutaneous injection. It is of particular interest, therefore, to study (1) the regional distribution of 5-HT and histamine in the skin of the rat, (2) the blueing of the skin of the rat after intravenous injections of vital blue dye and histamine liberators, (3) the blueing of the skin of the rat whose skin histamine has been first depleted, (4) the blueing of the skin of the rat whose skin 5-HT has been first depleted, and (5) the blueing of the skin of the rat after pre-treatment with specific antagonists.

METHODS

Groups of four or more albino rats (100–150 g) were used in all experiments. The skin and its adjacent subcutaneous connective tissue was removed from various regions and divided into two parts. One part was extracted with acetone and tested for 5-HT activity, and the other was extracted with trichloroacetic acid and tested for histamine activity. Extraction procedures, bioassay methods and identification of the two amines have been described in detail (Parratt & West, 1957*a*). All values in the tables refer to the bases and are the means of at least two separate estimations of each region.

For the capillary permeability tests, each rat was lightly anaesthetized with ether and injected intravenously with a mixture of Evan's blue dye (20 mg/kg) and one of the two histamine liberators, compound 48/80 or polymyxin B (1 mg/kg). To deplete the skin of its histamine before challenge with the dye and liberator, 5 doses of polymyxin B were given intraperitoneally over 3 days (Parratt & West, 1957*b*) and tests were made 24 hr later. To deplete the skin of its 5-HT before challenge, daily doses of reserpine (10 mg/kg) were given intraperitoneally for 2 days and tests were made 24 hr later. The antagonists used were mepyramine maleate, 2-bromo-lysergic acid diethylamide (BOL 148), promethazine hydrochloride (3277 RP), chlorpromazine hydrochloride (4560 RP) and 3-methoxy-(3′-dimethylamino-2′-methyl-propyl)-10-phenothiazine hydrochloride (7044 RP). Mepyramine is a specific antagonist of histamine, BOL 148 of 5-HT, whilst the other antagonists used are not specific but possess both antihistamine and anti-5-HT properties. These were administered intravenously 30 min before the challenge with the dye and liberator. The extent of the blueing and swelling of the skin areas was recorded visually on a relative scale, similar to that used for the assessment after local injections (Parratt & West, 1957*c*).

RESULTS

The regional distribution of 5-*HT and histamine in rat skin*

The histamine content of the skin varied greatly in different regions and the regional differences were substantially the same in all the groups of animals tested. In Table 1 the histamine content is given in μg/g. The figures in the third column were obtained by expressing the individual values in terms of the value for the abdominal skin taken as unity. These values are also charted in the left-hand side of the diagram of the body surface in Fig. 1.

The corresponding 5-HT values are also shown in Table 1 and Fig. 1 (right side). It will be noted that 5-HT occurs in all parts of the skin studied, the highest amounts being in the lips and nose, throat, base of ears and tongue.

There appears to be a relationship between the 5-HT content and histamine content though there are exceptions (for example, bristle area, scrotum, dorsal skin of hind legs and forelegs, the margin of the ears and the margin and base of the tail). Such a relationship favours an association of 5-HT with tissue mast cells (which hold most of the skin histamine) but this is unlikely since prolonged treatment with polymyxin B depletes the tissue of its histamine but leaves its 5-HT almost unchanged.

Induction of blueing by compound 48/80 *or by polymyxin B*

The injection of Evan's blue dye alone produced during the first hour only a faint grey-blue coloration of the skin, providing a background on which the blueing produced by the histamine liberators was readily detectable. Within 2 min of injecting 48/80, a faint blueing appeared on the eyelids and round the nostrils and lips. Within 4 min of the injection, there was intense blueing of these regions and faint blueing started at the base of the ears, tail and bristles, and round the nipples and anus. The full extent of the blueing was complete in about 30 min. The regions that were most deeply stained were those in which the dye first appeared. Though the ear margin, dorsal skin of the feet

TABLE 1. Histamine and 5-HT contents of various regions of the skin of the rat

Site	Histamine (μg/g)	Histamine (dorsal abdomen taken as unity)	5-HT (μg/g)	5-HT (dorsal abdomen taken as unity)	Histamine: 5-HT ratio	
					10–30	Over 30
Lips and nose	72	3·3	4·36	3·3	16	—
Scrotum	63	2·8	1·40	1·1	—	45
Bristle area	62	2·8	2·05	1·6	—	31
Tongue, female	55	2·4	2·90	2·2	19	—
Submental	54	2·4	2·10	1·6	26	—
Hind legs, dorsal skin	52	2·4	1·31	1·0	—	40
Throat	48	2·2	4·20	3·2	11	—
Eyelids	48	2·2	2·20	1·7	22	—
Forelegs, dorsal skin	48	2·2	1·32	1·0	—	36
Ear margin	48	2·2	1·40	1·1	—	34
Cheek	43	2·0	2·20	1·7	20	—
Ear base	41	1·9	3·20	2·5	13	—
Groin	38	1·7	1·80	1·4	21	—
Tongue, male	35	1·6	1·91	1·5	18	—
Nipples	33	1·5	2·47	1·9	13	—
Ventral abdomen	30	1·4	2·60	2·0	12	—
Anal margin	24	1·1	2·10	1·6	11	—
Dorsal abdomen	22	1·0	1·30	1·0	17	—
Tail margin	19	0·9	0·43	0·3	—	45
Tail base	19	0·9	0·36	0·3	—	53

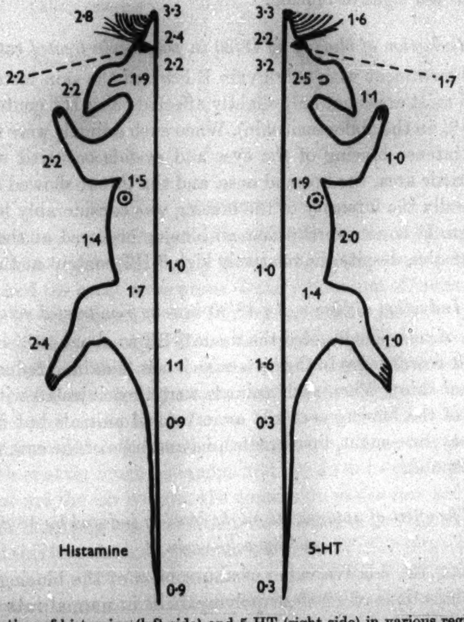

Fig. 1. Distribution of histamine (left side) and 5-HT (right side) in various regions of the skin of the rat. The figures are those given in columns 3 and 5 of Table 1 and represent values for each region taking that of the dorsal abdomen as unity.

and scrotum contain much histamine, very little blueing was seen in these regions. It will be apparent from Table 1 that these regions contain relatively less 5-HT than the other regions studied, and it is known that 48/80 releases both 5-HT and histamine (Bhattacharya & Lewis, 1956). It is possible therefore that the blueing of the tissues after 48/80 and vital blue dye results from the release of both 5-HT and histamine, both amines being necessary for the full effect. An alternative explanation why blueing does not occur in the ear margin, skin of the feet and scrotum may be that the sudden release of large quantities of histamine from the tissues lowers the blood pressure so rapidly that oedema of the tissues of certain extremities cannot occur. *Intraperitoneal* injections of 48/80 produce some blueing of these three regions, presumably because the blood pressure falls slowly and steadily and oedema can occur when capillary permeability is increased.

When polymyxin B was used as the histamine liberator, the order of blueing was different but the end result was similar to that seen after 48/80. The base of the ears always showed intense blueing first, followed by lips, nose, bristle area, eyelids, nipples and anus. It is not yet clear why the base of the ears reacts rapidly after polymyxin B. The ear margin, skin of the feet and scrotum again showed few signs of blueing.

Induction of blueing by 48/80 *in polymyxin-treated rats*

Prolonged treatment with polymyxin B lowered the skin histamine to less than 10% in most areas but only slightly affected the 5-HT content (maximal reduction 25% in the abdominal skin). When such animals were then injected with 48/80, intense blueing of the eyes and eyelids occurred within 2 min. Later, the bristle area, the lips and nose, and the throat showed some blueing though generally the intensity of the blueing was considerably less than that normally seen. It is noteworthy that no blueing occurred at the base or the margin of the ears, despite the relatively high 5-HT content at the base of the ears.

Induction of blueing by 48/80 *in reserpine-treated rats*

Two doses of reserpine lowered the tissue 5-HT to about 10% of the control level with but few changes in the histamine levels (maximal reduction 26% in the abdominal skin). When such animals were then injected with 48/80, the distribution of the blueing occurred as in normal animals but it was not so intense. The eyelids, snout, lips, bristle area and base of the ears were the first regions to respond.

The effect of antagonists on the blueing induced by 48/80 *and by polymyxin B*

For assessing the relative value of antagonists of the blueing induced by intravenous injections of 48/80 or polymyxin B in normal rats with Evan's blue dye in their circulation, visual records of the facial regions were made, since the feet did not exhibit blueing. Mepyramine in a dose of 8 mg/kg provided a 75 % protection against blueing induced by 48/80 but only a 28 % protection against that induced by polymyxin B (Table 2). Promethazine was more potent against 48/80 as well as polymyxin B than mepyramine. Of the five antagonists used, chlorpromazine was the most efficient inhibitor of the responses, doses as low as 0·5 mg/kg exerting a definite effect on the blueing induced by 48/80. In all cases blueing induced by polymyxin B was more difficult to inhibit than that induced by 48/80.

TABLE 2. The effect of antagonists on the blueing induced in rats by intravenous doses (1 mg/kg) of 48/80 or of polymyxin B. Responses measured in terms of the maximal (100 %) found in saline-treated rats

		Blueing induced by	
Antagonist	Dose (mg/kg)	48/80	Polymyxin B
Mepyramine	2	100	100
	4	80	100
	8	25	72
Promethazine	2	50	65
	4	0	20
	8	0	0
Chlorpromazine	0·5	55	100
	1	0	100
	2	0	70
7044 RP	0·5	100	90
	1	60	60
	2	0	20
BOL 148	1	90	100
	2	45	100
	4	0	90

DISCUSSION

The systemic study by Feldberg & Miles (1953) of the histamine content of the skin of the guinea-pig showed clearly that a definite relationship exists between this content and the areas more prone to the production of increased capillary permeability by compound 48/80. The present results indicate that a similar relationship exists in the rat. Thus, the eyelid, lips and nose, bristle area, nipples, base of ear and anal regions are sites where increased capillary permeability, as manifested by oedema and by exudation of circulating dye, most readily occurs under the influence of the two histamine liberators used, and these regions generally are rich in histamine. However, there are three regions which contain much histamine and yet do not exhibit the full phenomenon. There are the ear margin, the dorsal skin of the feet and the scrotum. They contain relatively less 5-HT than do other areas with similar histamine content, and this fact alone may account for the lack of blueing of such regions. For example, signs of increased capillary permeability in the ear are confined to the base which contains more than twice the concentration of 5-HT found in the margin. It is also well known that 5-HT in the rat is a much more efficient agent in increasing capillary permeability than is histamine.

There is another kind of discrepancy between proneness to increased capillary permeability and histamine content of the skin region. The range of intensity of blueing or of oedema formation in different regions is greater than the range of histamine concentration. On the evidence of blueing alone, it could be said that no histamine was released in the abdominal skin of the rat by 48/80 or by polymyxin B, although the histamine it contains (20–30 μg/g) is more than sufficient to produce an intense local blueing when released by these liberators injected intradermally. The vascularity of the skin, the arteriovenous anastomoses, the degree of sensitivity of the skin capillaries to endogenous histamine, or even the readiness with which extravascular tissue is able to accumulate the dye, may thus be other contributory factors determining the regional pattern of increased permeability of the skin capillaries seen after histamine liberators.

In the rat depleted of its histamine by polymyxin B, blueing, though reduced in intensity, still occurs after the injection of dye and 48/80, with one exception—that at the base of the ears. The reason for this failure is not clear. After each dose of polymyxin B used to produce the histamine depletion, local swelling at the base of the ears always occurred, even when the histamine content of this region was reduced to minimal levels (10 % of the control value). Since polymyxin B by itself does not possess a vasodilator action when tested in perfusion experiments, it is suggested that a vasodilator substance, in addition to histamine, is involved in the vasodilator effect of polymyxin B when injected into the rat, but that 48/80 fails to release this substance.

When 5-HT-depleted rats are used, blueing of the skin after 48/80 is partly suppressed, confirming that 5-HT, as well as histamine, participates in the mechanism by which 48/80 increases capillary permeability in the rat (Sparrow & Wilhelm, 1957). Further support for this view is supplied by the results using specific antagonists. Some anti-5-HT agents are as efficient as some antihistamine agents in reducing the blueing reaction of 48/80, whilst others possessing both anti-5-HT and antihistamine properties are even better. Thus, when 48/80 is injected into normal rats to produce the blueing reaction, the most potent of the five antagonists used here are promethazine, chlorpromazine and 7044 RP, as these three substances possess both anti-5-HT and antihistamine properties. The specific antihistamine substance, mepyramine, and the specific anti-5-HT agent, BOL 148, are much weaker in this respect. Doses of all the inhibitors have to be considerably increased to obtain any reduction of the blueing following polymyxin B injection.

Usually the degree of swelling (oedema) and of blueing (leakage of the plasma proteins into the tissue spaces) go hand in hand, though the mechanisms involved may be different. Gözsy & Kátó (1956), for example, have suggested that, whereas oedema is a record of the increase in capillary permeability, the blueing is due to the phagocytic action of the endothelial cells of the blood vessels. In the present experiments intravenous doses of 48/80 produced some swelling of the extremities but no blueing, so that the two phenomena may not be directly related to each other. Likewise, Sparrow & Wilhelm (1957) showed that on occasion it was easier to reduce the intensity of the blueing than it was to reduce the diameter of the lesions induced by 48/80.

We have assumed that tissue mast cells contain most of the skin histamine, and in the rat these cells are known to be disrupted by histamine liberators such as compound 48/80 or polymyxin B. However, in the guinea-pig, the effects of 48/80 on tissue mast cells are minimal, since many lose granules but few disrupt (Mota & Vugman, 1956). Thus the histamine release in guinea-pigs noted by Feldberg & Miles (1953) may well be the result of degranulation of the mast cells, since it is known that a significant release of histamine can occur in the rat without gross disruption of mast cells.

SUMMARY

1. The regional distribution of 5-HT and histamine in the skin of the rat has been determined. In most areas of skin there is a relationship between the 5-HT and histamine contents.

2. The areas more prone to the production of increased capillary permeability by histamine liberators are rich in both histamine and 5-HT. Other areas containing histamine but less 5-HT exhibit this response weakly.

3. The swelling and blueing after intravenous injections of vital blue dye and histamine liberators are reduced, but not entirely prevented, by depletion of skin 5-HT or histamine. A third permeability factor may thus be involved.

4. Antagonists possessing both anti-5-HT and antihistamine properties are potent inhibitors of the alteration in capillary permeability produced by histamine liberators.

5. It is suggested that 5-HT, as well as histamine, plays a role in the production of increased capillary permeability by histamine liberators.

We wish to thank Dr P. Viaud for the compound 7044 RP.

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THE REACTIONS OF THE CAPACITY BLOOD VESSELS OF THE HUMAN HAND AND FOREARM TO VASO-ACTIVE SUBSTANCES INFUSED INTRA-ARTERIALLY

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All blood vessels both carry and hold blood. All exhibit both resistance to the flow of blood and capacity to contain blood. In some the resistance is the conspicuous feature, and is subject to variation. In others the capacity is considerable and the resistance to flow is small. Little is at present known about the control of the capacity vessels in the human subject. The methods available for study of the whole limb vasculature enable the total resistance and total capacity of the limb to be measured, but do not distinguish between the contributions of the various anatomical groups of vessels to these totals. In the present study a distinction is made between the capacity of low-pressure vessels, which are emptied when the limb is raised and subjected to an external hydrostatic pressure, and the capacity of the higher-pressure vessels which are not emptied in these circumstances. The reactions of these two groups of vessels to intra-arterial infusions of adrenaline, noradrenaline, 5-hydroxytryptamine and histamine have been investigated.

If, as a first approximation, and over short periods of time, the extravascular volume of the limb is assumed to be constant, then changes in the volume of the raised limb exposed to hydrostatic pressure may be ascribed mainly to changes in the behaviour of the high-pressure capacity vessels. Changes in the distensibility of the limb on application of venous congestion, that is, changes in the form of the pressure–volume curve, may be ascribed mainly to changes in the behaviour of low-pressure capacity vessels.

METHODS

The investigation was carried out on fifteen healthy young subjects at a room temperature of 23 ± 1° C. The subject lay supine on a couch with the left arm abducted and raised about 10° above the horizontal so that the forearm was just above heart level; and the forearm or hand was enclosed in a water-filled, stirred plethysmograph at 35° or 32° C respectively (Greenfield, 1954). A pneumatic cuff surrounded the arm proximal to the plethysmograph. Increases in the volume of the part displaced water from the plethysmograph through a constant temperature reservoir immersed in the water in the outer jacket of the plethysmograph into a burette calibrated in millilitres. The position of the burette was continuously adjusted to keep the meniscus at a reference level 10 cm above the centre of the part and the burette reading was noted at either 15 or 30 sec intervals. This was done both under resting conditions and during graded venous congestion. A needle was inserted into the brachial artery just proximal to the elbow and a solution of sodium chloride 0·9% (w/v) was infused at a rate of 4 ml./min by a mechanically driven syringe. When vasoactive substances were infused the dose for 1 min was made up in 4 ml. of saline. The substances infused were L-noradrenaline hydrogen tartrate (Levophed, Bayer Products), L-adrenaline hydrogen tartrate (British Drug Houses), 5-hydroxytryptamine creatinine sulphate (Sandoz Products) and histamine dihydrogen phosphate (British Drug Houses). Ascorbic acid (Roche) in a concentration of 1:50,000 was added to the adrenaline and noradrenaline solutions to delay oxidation (Gaddum, Peart & Vogt, 1949).

Experimental procedure

The subject rested for 30 min before any observations were made, and the intra-arterial infusion was then started. The venous pressure in the forearm or hand was raised by inflating the pneumatic cuff around the arm. It was found that until the cuff pressure had risen to a level usually between 10 and 20 mm Hg above atmospheric (the 'starting pressure'), which remained constant in any one experiment, no change in volume occurred. The volume increases brought about by pressures 20, 40 and 60 mm Hg above this 'starting pressure' were observed. When the congesting pressure was applied, the volume increased rapidly at first and then more slowly. The final reading was taken when the volume became steady or the rate of increase fell below 0·15 ml./100 ml. tissue in the plethysmograph/min. The cuff pressure was then returned to atmospheric and further congesting pressures were not applied until the volume was again steady. The volume increases so obtained were found to be reproducible on repeated imposition of the same congesting pressure. The vasoactive substance was then infused and base-line readings were continued for 2–5 min to allow any change in resting volume to be observed. When the readings were steady the same congesting pressures as before were applied and the volume increases noted. The drug infusion was then stopped and the saline resumed, and time allowed for a steady base line to be attained; this base line did not always coincide with that before the drug infusion. In most experiments this procedure was repeated using larger doses of the drug.

Presentation of results

The record of volume changes observed during a typical experiment is illustrated in Fig. 1. The results were calculated as follows. Changes in the content of the high-pressure capacity vessels were estimated by comparing the base-line readings of the water level at a time half way through the infusion period with the mean of the last three volume readings before the infusion started and the first three readings when the base line volume had regained a steady level following the infusion (Fig. 1*a*). The capacity of the low-pressure vessels was estimated by measuring the difference between the base-line readings and the final congested level (Fig. 1*b*). The volume of the forearm or hand enclosed in the plethysmograph was afterwards measured by water displacement, and all volume changes were expressed in ml./100 ml. of tissue.

RESULTS

Experiments on both the forearm and the hand were carried out with each of the substances studied, and the results of all experiments are contained in Table 1. The results of a typical experiment with each substance are illustrated graphically in Fig. 2.

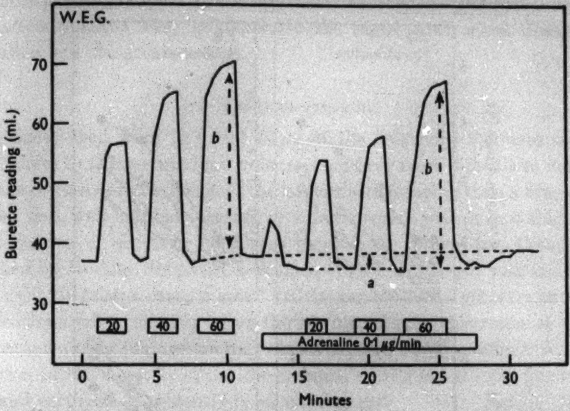

Fig. 1. Volume changes in millilitres occurring during a typical experiment on W.E.G. During the second run, adrenaline was infused intra-arterially at a rate of 0·1 μg/min. Alteration in content of the high-pressure vessels was measured as described in the text and as illustrated at *a*. The low-pressure capacity was measured as illustrated at *b*.

TABLE 1. Change (%) in volume of forearm or hand during saline and drug infusion into the brachial artery

		Saline				Noradrenaline											
						0·1 μg/min				0·4 μg/min				1 μg/min			
Congesting pressure (mm Hg)	...	0	20	40	60	0	20	40	60	0	20	40	60	0	20	40	60
Forearm	J.Q.	0	1·8	3·0	4·0	– 0·7	0·5	1·7	3·0	– 1·3	0·5	1·4	2·8	– 2·0	0·25	1·3	2·3
	E.Rt.	0	0·6	2·6	3·7	– 0·2	0·4	1·5	2·6	– 0·4	0·1	0·5	1·5	– 1·0	0·4	0·8	1·8
	E.Ry.	0	2·4	4·0	4·9	0	2·5	3·8	4·6	– 0·4	1·2	2·5	3·6	– 0·5	0·8	2·0	2·8
Hand	D.G.	0	1·5	2·0	2·5	– 1·1	0·5	0·9	1·5	– 1·2	0·3	0·7	1·0	—	—	—	—
	P.S.	0	1·6	2·1	2·8	– 1·0	1·1	1·7	1·8	– 1·4	0·7	1·4	1·5	—	—	—	—
						Adrenaline											
						0·1 μg/min				0·4 μg/min				1 μg/min			
Forearm	E.B.	0	1·7	3·4	4·0	– 0·4	1·5	2·3	3·5	– 0·6	0·6	1·6	2·1	– 0·8	0·5	1·3	2·3
	E.W.	0	1·2	2·1	2·8	– 0·6	0·9	2·0	2·7	– 0·7	1·1	1·9	2·5	– 1·3	0·8	1·3	2·0
	W.E.G.	0	3·1	4·7	5·3	– 0·4	2·8	3·5	5·1	– 1·1	1·9	3·3	5·1	– 1·6	1·6	2·7	4·0
Hand	E.B.	0	1·3	1·6	2·0	– 1·6	0·6	0·9	2·0	– 1·1	0·4	0·7	1·1	—	—	—	—
	J.McC.	0	1·5	2·3	2·6	– 0·8	1·0	1·3	2·0	– 1·3	0·6	0·7	0·8	—	—	—	—
						5-Hydroxytryptamine											
						1 μg/min				4 μg/min				16 μg/min			
Forearm	H.O'K.	0	3·5	4·5	6·0	– 0·2	0·7	1·3	3·2	– 0·3	0·7	1·3	2·5	– 0·8	1·0	1·4	2·8
	J.B.	0	1·2	4·2	5·9	+ 0·2	2·5	3·5	4·3	– 0·3	1·0	1·5	2·7	– 0·1	0·5	1·3	1·8
	A.D.M.G.	0	1·3	2·4	3·4	0	0·8	1·4	2·1	0	0·6	0·8	1·5	0	0·3	0·4	0·8
Hand	M.L.	0	1·6	3·2	3·6	0	0·9	0·9	1·5	0	0·7	0·7	0·7	—	—	—	—
	J.B.	0	1·6	3·1	3·9	0	0·6	0·8	0·9	—	—	—	—	—	—	—	—
						Histamine											
						1 μg/min				2 μg/min				5 μg/min			
Forearm	W.E.G.	0	1·5	4·0	4·8	+ 0·4	1·8	3·8	4·8	+ 0·5	2·0	3·7	4·5	+ 0·7	1·7	3·8	4·6
	H.McK.	0	2·3	4·5	5·5	+ 0·5	2·5	4·2	5·2	+ 0·6	2·1	4·2	5·4	+ 0·4	2·5	4·5	4·9
	J.B.	0	2·7	4·2	5·0	+ 0·7	2·8	3·9	5·1	+ 1·1	2·5	3·7	5·0	+ 1·7	2·3	3·5	4·8
Hand	A.D.M.G.	0	1·6	2·1	2·6	+ 0·6	1·3	2·1	2·5	+ 0·8	1·2	2·0	2·5	—	—	—	—
	J.Q.	0	2·0	3·5	4·5	+ 1·2	2·5	3·2	3·9	+ 1·4	2·8	3·4	4·2	—	—	—	—

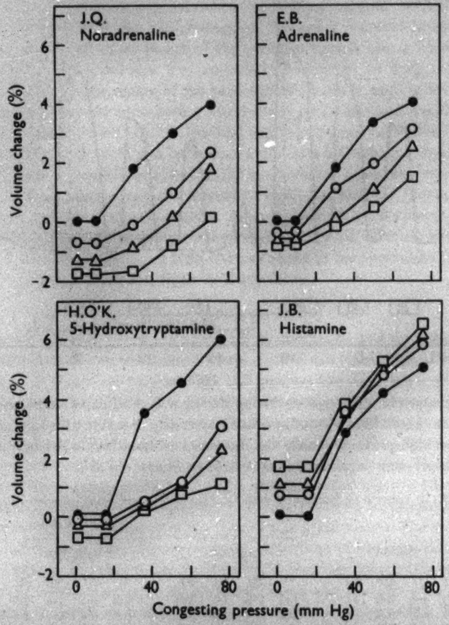

Fig. 2. Typical pressure–volume curves illustrating the effect of intra-arterial infusions of noradrenaline, adrenaline, 5-hydroxytryptamine and histamine on the high- and low-pressure capacity vessels of the forearm. Change in volume of the high-pressure capacity vessels is illustrated by the height of the curve before the 'starting pressure' is reached. ● indicates the run in all experiments in which saline alone was infused; other doses thus (μg/min):

	○	△	□
Noradrenaline and adrenaline	0·1	0·4	1·0
5-hydroxytryptamine	1	4	16
Histamine	1	2	5

Noradrenaline and adrenaline

These substances were infused in doses of 0·1, 0·4 and 1·0 μg of the base/min. With each subject there was a reduction in the resting volume of the part following the start of the infusion which indicated mainly a reduction in the content of the high-pressure vessels. When the venous congestion was applied the amount of blood which could be collected in the part was reduced, which indicated that low-pressure capacity was also reduced. Similar results were obtained for the hand and forearm and in all experiments the effects were more marked as the dose was increased. While the results with noradrenaline and adrenaline were qualitatively the same, with equal dosage noradrenaline had the greater effect.

5-*Hydroxytryptamine*

The doses used were 1, 4 and 16 μg of the creatinine sulphate salt/min. The changes in the resting limb volume were here more difficult to interpret, since the intra-arterial infusion of the larger doses for more than a few minutes resulted in a well-marked increase in volume which was presumably due to the formation of oedema. This large increase in volume has already been described by Roddie, Shepherd & Whelan (1955). However, with the smaller doses used there was often a small and transitory initial increase in volume followed by a fall to slightly below the resting volume; this response was not as constant nor as marked as that observed with noradrenaline. A marked reduction in the capacity of the low-pressure vessels in both the forearm and the hand occurred consistently in all experiments.

Histamine

Histamine acid phosphate in doses of 1, 2 and 5 μg of the salt/min caused in all cases an increase in the base-line volume of the part, the size of the increase becoming more marked with increasing doses. The capacity of the low-pressure vessels, however, was unaltered by this infusion.

DISCUSSION

High-pressure capacity vessels

In these experiments, two measurements have been made, of which the first is of changes in the resting volume of the hand or forearm during the infusion of vasoactive substances. It has been shown that this volume can either increase or decrease, and since the volume changes rapidly on starting the infusion and almost as rapidly reverts towards its former level when the infusion is ended, it is assumed that this change in volume is brought about by an alteration in the amount of blood in the part. Because the part is raised above heart level and exposed to an external pressure of 10 cm of water, the low-pressure capacity vessels are largely drained of blood, and so this change in base-line volume must be due to an alteration in the content of the uncollapsed or higher-pressure vessels. While these high-pressure capacity vessels cannot be anatomically defined, it is probable that they are 'prevenous' and include those vessels the reactions of which largely determine the resistance to flow (resistance vessels).

It is of interest to note that both with adrenaline and 5-hydroxytryptamine there was often, immediately following the start of the infusion, a slight and transient increase in volume which corresponded roughly with the transient increase in flow seen in the forearm with these substances given intra-arterially (Duff & Swan, 1951; Roddie *et al.* 1955). Although this suggests that the resistance vessels contribute to the high-pressure capacity, they by no means represent a major component of it. High-pressure capacity and resistance do not always change in parallel. For example, adrenaline reduces the high-pressure capacity both in the hand and the forearm, but while it increases the resistance to flow in the hand, on average it does not change the resistance in the forearm (Duff & Swan, 1951).

The content of the high-pressure capacity vessels is reduced by noradrenaline but is increased during histamine infusions.

The effect of 5-hydroxyptamine on the high-pressure capacity is difficult to interpret because following the infusion of even small doses the volume returns only very slowly towards the base line, and following the infusion of larger doses the volume remains above the base line for a very long time, presumably because of oedema formation, which can amount to 3% with continued infusion of a dose of 16 μg/min (Roddie *et al.* 1955). The reduction in the content of the high-pressure vessels is therefore partly obscured by this over-all increase in volume.

The effect of intra-arterial infusions of the above substances on resistance vessel calibre has been extensively investigated by measurements of blood flow. Noradrenaline, in doses of 0·1 and 0·125 μg/min respectively, has been found to cause a reduction in forearm and hand flows to one-third (Barcroft, Gaskell, Shepherd & Whelan, 1954; Duff, 1955). The effect of intra-arterial adrenaline is somewhat variable, but on average in doses of 0·125 μg/min it does not change, or slightly reduces, forearm flow (Duff & Swan, 1951), while 0·5 μg/min reduces hand blood flow to one-third (Swan, 1951). 5-Hydroxytryptamine in the doses used in our experiments reduces the blood flow through the forearm to between one half and one quarter, and through the hand to between one quarter and one tenth (Roddie *et al.* 1955). Histamine in a dose of 4 μg/min increases the forearm and hand flows three- and sixfold respectively (Duff, Greenfield, Shepherd & Thompson, 1953).

Low-pressure capacity vessels

The second measurement made is of the amount of blood which can be collected in the part by graded venous congestion as applied by a pneumatic cuff. The 'starting' pressure which was applied to the cuff was determined by 'titrating' the cuff pressure against the volume change, and was critical. This was the pressure necessary to raise the intraluminar pressure in the collapsed vessels in the plethysmograph to a pressure slightly greater than the tissue pressure, which has here been augmented by an external hydrostatic head of 10 cm of water. With increased congesting pressure the previously collapsed vessels filled up and their behaviour largely determined the pressure-volume curve.

The results of the present experiments show that intra-arterial infusions of noradrenaline, adrenaline and 5-hydroxytryptamine cause the low-pressure capacity vessels to become less distensible while infusion of histamine does not alter the capacity of these vessels. As in the case of the high-pressure capacity vessels, these low-pressure vessels have no precise anatomical definition but can be described as being mainly 'venous'.

These results are in broad agreement with those of previous workers in this field. Donegan (1921) reported that in animal experiments adrenaline constricted the superficial and mesenteric veins, and Fleisch (1931) confirmed this in cats. In the human subjects Lewis (1924) showed that the venules in the skin contracted following local application of adrenaline, and more recently Burch & Murtadha (1956) have demonstrated that noradrenaline introduced into an isolated segment of superficial forearm vein will cause an intense constriction. Reid (1952) showed that 5-hydroxytryptamine injected subcutaneously around a vein caused constriction and Roddie *el al.* (1955) reported a reduction in the amount of blood that could be accommodated in the hand during intra-arterial infusions.

While noradrenaline and 5-hydroxytryptamine, which are constrictor to the resistance vessels, also produced a reduction in low-pressure capacity, histamine, which is a resistance-vessel dilator, failed to cause an increase in this capacity. This finding is in keeping with that of Kidd & Lyons (1958) who demonstrated a fall in low-pressure capacity with local cold but no increase with local heating, general heating or exercise of the limb muscles, all of which dilate resistance vessels. Observations on the pressure in functionally isolated segments of human veins *in situ* in the forearm (Duggan, Love & Lyons, 1953; Page, Hickam, Sieker, McIntosh & Pryor, 1955; Burch & Murtadha, 1956) and observations on small balloons inserted into veins on the dorsum of the hand (Connolly & Wood, 1954) and in limb veins of animals (Salzmann & Leverett, 1956; Salzmann, 1957) have shown that veins can contract in response to local and general stimuli; a dilatation has not been demonstrated in normal subjects. Thus although large veins represent only one component of the total low-pressure capacity system, it is of interest that their reactions parallel those of the whole system.

In summary, while the low-pressure, and perhaps predominantly venous, vessels can reduce their capacity under certain strong stimuli, it has not been shown that these vessels can increase their capacity from ordinary resting levels with any of the stimuli tested. It is suggested that in the comfortably warm subject these vessels are normally at their maximal distensibility and that the amount of blood they contain is determined by the transmural pressure acting on their walls, and not by the reactivity of the walls.

SUMMARY

1. In warm recumbent subjects with the arm raised about 10° above the horizontal, measurements have been made of (*a*) the volume of the hand or forearm exposed to an external pressure of 10 cm of water, and (*b*) the increase in this volume during graded venous congestion of up to 60 mm Hg.

2. Changes in (*a*) were considered to be mainly due to changes in the higher-pressure ('prevenous') capacity vessels, and changes in (*b*) mainly due to changes in the lower-pressure ('venous') capacity vessels.

3. Observations were made when noradrenaline, adrenaline, 5-hydroxytryptamine and histamine were infused into the brachial artery.

4. Reactions in the hand and forearm were found to be qualitatively similar in all cases.

5. Both noradrenaline and adrenaline in doses of 0·1, 0·4 and 1·0 μg/min caused a reduction in both high- and low-pressure capacity.

6. 5-Hydroxytryptamine in doses of 1, 4 and 16 μg/min caused a well-marked reduction in low-pressure capacity. The effect on the high-pressure capacity was partly obscured by a large and persistent increase in volume which was presumably due to oedema formation.

7. Histamine in doses of 1, 2, and 5 μg/min increased the content of the high-pressure capacity vessels, but did not alter low-pressure capacity.

We are grateful to Sandoz Products Ltd. for supplies of 5-hydroxytryptamine.

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THE DISTENSIBILITY OF THE BLOOD VESSELS OF THE HUMAN CALF DETERMINED BY GRADED VENOUS CONGESTION

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It has been well established that the veins in the human subject react to general and local stimuli (Doupe, Krynauw & Snodgrass, 1938; Duggan, Love & Lyons, 1953; Connolly & Wood, 1954; Burch & Murtadha, 1956). Of importance, however, to the general economy of the circulation is whether these reactions can influence the amount of blood held in the capacity vessels of a part under conditions of normal life. A recent study of the capacity vessels of the human calf has suggested that some of the stimuli which are known to dilate the blood vessels chiefly concerned with providing resistance to blood flow through the calf do not alter the ability of the calf blood vessels to hold blood (Coles, Kidd & Moffatt, 1957).

The present investigation was designed to study the reactions of the capacity vessels of the calf under similar circumstances using a simpler method of wider application.

METHODS

The subjects were eight healthy young men; the subject lay on a couch with one thigh supported so that the hip and knee joints were slightly flexed. The calf was enclosed in a plethysmograph (Greenfield, 1954), containing, unless otherwise stated, stirred water at 35° C, and the axis of the calf was 16 cm above the phlebostatic level (Burch, 1950). Pneumatic cuffs surrounded the leg immediately distal to the plethysmograph and just proximal to the knee joint. When a congesting pressure was applied to the thigh cuff, the calf increased in volume and displaced water from the plethysmograph, through a constant temperature reservoir which stood in the outer jacket of the plethysmograph, into a burette calibrated in ml. The water level in the burette was adjusted to be read at a constant reference level 12 cm above the upper surface of the calf and 28 cm above the phlebostatic level.

When the subject had rested for 30 min, the pneumatic cuff distal to the plethysmograph was inflated to 200 mm Hg and the blood flow through the calf was recorded by conventional venous occlusion plethysmography. The plethysmograph was then connected to the burette and direct readings of the water level were made at 30 sec intervals, while the thigh cuff was inflated successively to pressures 10, 20, 30, 40, 50, 60, 70 and 80 mm Hg above atmospheric. At the lower pressures the volume changes were slight and irregular. At the higher pressures the rate of volume increase was rapid for the first 2–3 min and then gradually diminished, eventually becoming very slow. The distended volume at any congesting pressure was taken as the volume attained when the rate of increase fell below 0·13 ml./100 ml. calf tissue/min, and the cuff pressure was then returned to atmospheric. The calf volume at the end of the congestion never differed from that at the beginning by more than 0·15 ml./100 ml. of tissue. The observed volume increase was expressed in ml./100 ml. of calf tissue and a pressure–volume curve was constructed.

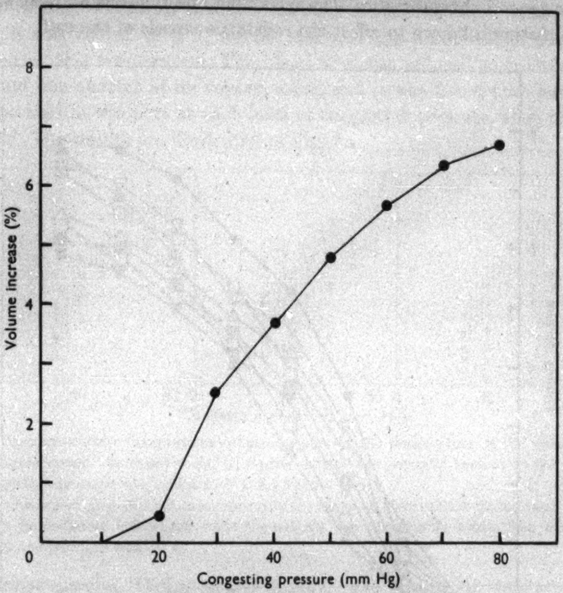

Fig. 1. A typical pressure–volume curve, demonstrating the percentage increase in calf volume with increasing calf pressure.

RESULTS

Fig. 1 illustrates the volume increase in ml./100 ml. of tissue in relation to the various congesting pressures used, in a typical experiment. It can be seen that there was no volume increase at 10 mm Hg and only a small increase at 20 mm Hg, but thereafter the increase was considerable for each 10 mm Hg increment in pressure. Two factors may account for the failure of the low pressure to cause any volume increase: first, the pressure in the cuff may not be fully transmitted to the vessels in the deeper parts of the limb, and secondly, the pressure in the veins enclosed in the plethysmograph must increase sufficiently to overcome the hydrostatic pressure acting on the surface of the leg before any blood can accumulate in them. With the lowest pressures results were not always reproducible, but with congesting pressures above 20 mm Hg the pressure–volume curve was reproducible and showed only slight variations from day to day.

Fig. 2 shows one pressure–volume curve from each of the eight subjects studied, and while there was some individual variation, the curves are similar in their general characteristics. The remaining observations all deal with the effects of stimuli known to affect the resistance vessels of the calf.

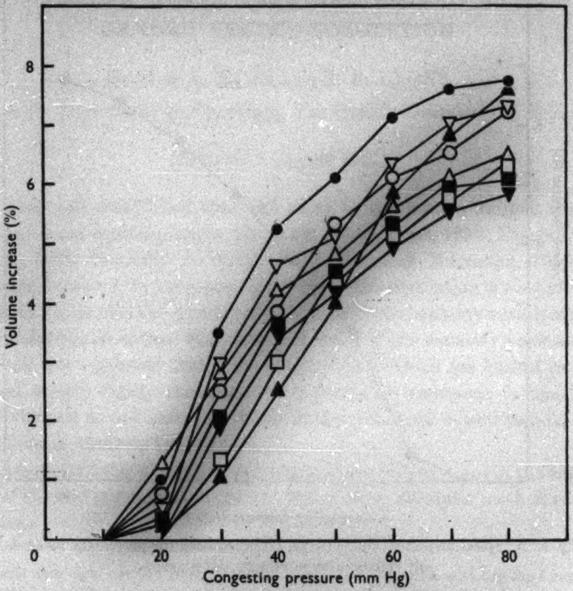

Fig. 2. Pressure–volume curves for each of the eight subjects tested.

Local heating. In these experiments standard pressure–volume observations were first made with the water in the plethysmograph at 35° C, as described above. The water was then rapidly heated to 44° C, and after 30 min further observations were made at 44° C. The blood flow was measured and found to be increased by 2–3 times, indicating dilatation of the resistance vessels. It was found that raising the temperature had very little effect on the amount of blood which could be held in the calf. The results are summarized in Fig. 3*a*.

General heating. After standard pressure–volume observations had been made, the subject was wrapped in blankets, hot water bottles were applied to the trunk and abdomen, and his arms were immersed in water baths at 43° C. This procedure causes a large increase in blood flow; by analogy with the forearm (Roddie, Shepherd & Whelan, 1956), this increase is presumed to be mainly through the skin blood vessels. Despite the observed increase of from 3–6 times in calf blood flow, there was little alteration in the distensibility of the calf blood vessels. The results are as summarized in Fig. 3*b*.

Local cooling. Following the standard observations the water in the plethysmograph was cooled to 6° C for 30 min and the observations were then repeated at this temperature. The blood flow was reduced to between one-third and one-quarter of its resting value, and it was found that less blood could be held in the part at each level of congesting pressure when the limb was cold. The results are illustrated in Fig. 3*c*.

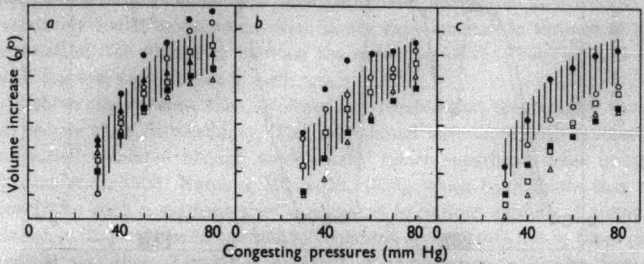

Fig. 3. Pressure–volume observations obtained while (*a*) the temperature of the water in the plethysmograph was raised to 44° C; (*b*) the subject was generally heated; (*c*) the water in the plethysmograph was cooled to 6° C for 30 min.

The hatched area in each case represents the range of observations on the same subjects made immediately beforehand while the subject was comfortably warm. The plethysmograph temperature was 35° C.

Muscular exercise. Cuff pressures of 30, 50 and 70 mm Hg were applied in succession, and at each pressure, when the calf volume had reached a steady level, the calf muscles were exercised by plantar flexion of the foot against a hinged board (Coles *et al.* 1957) 30 times a minute for 1 min. The cuff pressure was maintained for a further period after the exercise before being returned to atmospheric. Control observations were then made, in which the exercise was omitted. Experiments were carried out on six subjects and the results were in all cases similar to those illustrated in Fig. 4. It may be seen that during the exercise there is a reduction in the congested calf volume; when, however, the exercise ceased the volume of the calf rapidly reverted to a level very close to that observed in the control experiments. The calf blood flow at this time was greatly increased, and it is inferred that the vessels chiefly concerned with resistance to flow were dilated.

The recorded reduction in volume during exercise is considered to be reliable since no change in volume was observed in other experiments when the circulation was arrested as completely as possible while the exercise was carried out.

The reduction was presumably due to the action of the muscle pump in expressing blood from the calf.

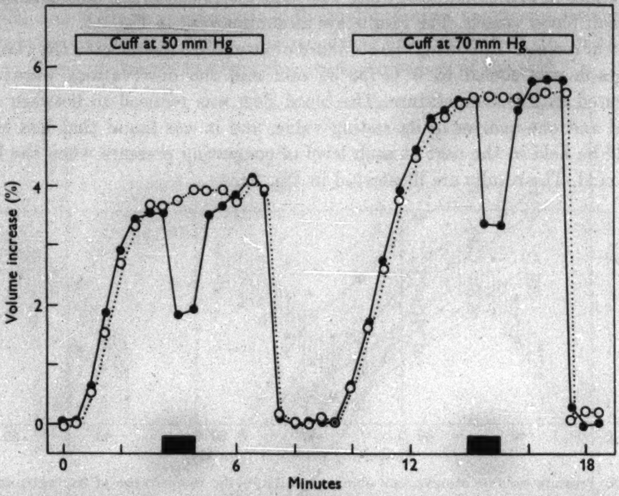

Fig. 4. Records of changes in volume of the calf. Curve ···○··· shows the effects of applying congesting pressures of 50 and 70 mm Hg for the periods shown. The same procedure was adopted in curve -●-, but, in addition, exercise (plantar flexion of the foot against a hinged board 30 times/min) was carried out for the period indicated, ■.

DISCUSSION

When a congesting pressure is applied to a leg raised above heart level, the volume of the calf increases as the previously collapsed capacity vessels fill up. Eventually the venous pressure distal to the cuff exceeds the cuff pressure; blood then escapes under the cuff and further volume increase is slight. This slight increase is due either to filtration of fluid into the tissues or to a relaxation of the capacity vessels. The size of the first rapid volume increase is taken as an index of the distensibility of the calf blood vessels at that particular congesting pressure.

In these experiments no measurements were made of the resting volume of blood in the limb or of the alterations in this under the various conditions studied. Since there were changes in blood flow, which implies that there were alterations in the calibre of the resistance vessels, it is possible that the resting volume of blood in the calf did vary. What is shown by these results is that the distensibility of the vessels which are collapsed in the elevated limb is unaltered by certain stimuli which dilate the resistance vessels. Cold, a stimulus which constricts the resistance vessels, on the other hand, reduces the distensibility of the capacity vessels.

These results are in agreement with those obtained for the calf, using local exposure to subatmospheric pressures as the distending force (Coles *et al.* 1957). Henry (1954) studied the effects of temperature on venous distensibility in the leg using positive-pressure breathing as the distending force. The present results confirm his observation that the amount of blood held in the leg is decreased by cooling. Henry, however, observed an increase on warming while we observed no change. The apparent discrepancy probably arises from differences in the 'normal' conditions in the two sets of experiments, since in Henry's the subject stood in a tank containing water and was exposed to a relatively cooler environment than in our experiments. In support of this explanation, the difference between the capacities of the 'hot' leg and the 'cold' leg was very similar in both sets.

In these experiments, then, evidence is presented that the capacity vessels can reduce their distensibility. This is in broad agreement with studies on functionally isolated though anatomically intact superficial veins in man (Duggan *et al.* 1953; Burch & Murtadha, 1956), which have shown that the pressure in such a segment rises in response to various stimuli. A point of interest is that neither in the isolated segment experiments nor in those here reported was there found any evidence of a relaxation in venous tone in normal subjects; it may well be that, in the comfortably warm subject in the horizontal position, the tone of the smooth muscle in the capacity blood vessel walls is fully relaxed.

SUMMARY

1. The volume increases of the human calf resulting from graded venous congestion have been measured plethysmographically in the raised leg of the recumbent subject.

2. These volume increases were reproducible for any one subject and when expressed as a percentage of the resting limb volume did not vary widely between the subjects tested.

3. Normal pressure–volume curves were determined, and any alteration in this curve was presumed to indicate an alteration in the distensibility of the capacity blood vessels.

4. This distensibility was unaltered by local heating of the limb, general heating or following muscular exercise; in all these conditions the blood flow through the part was greatly increased.

5. The distensibility was reduced by severe local cooling.

We wish to acknowledge the help and advice afforded to us by Professor A. D. M. Greenfield during this project.

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THE EFFECT OF VARIATION IN THE CONCENTRATION OF INDIVIDUAL EXTRACELLULAR ELECTROLYTES ON THE RESPONSE OF THE SHEEP'S PAROTID GLAND TO NA+ DEPLETION

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It has been shown (Denton, 1956, 1957 *b*) that withdrawal of the supplementary Na^+ intake from a sheep with a parotid fistula results in progressive Na^+ depletion, and deficits of 500–1000 m-equiv of Na^+ occur. Na^+ depletion of this degree causes the parotid salivary $Na^+:K^+$ ratio to fall from a normal value of 18·0 (180:10 m-equiv/l.) to as low as 0·06 (10:180 m-equiv/l.). Restoration of the dietary $NaHCO_3$ supplement, in an amount sufficient to correct the Na^+ depletion, causes the $Na^+:K^+$ ratio in the parotid saliva to return to normal.

Recent work in this laboratory, aimed at elucidating the mechanism of this striking phenomenon, has established the following facts:

(i) The fall of the salivary $Na^+:K^+$ ratio during Na^+ depletion is associated with a 5–20 m-equiv/l. decrease of plasma Na^+ concentration (Denton, 1957 *b*).

(ii) A local parotid mechanism associated with a decrease of secretion rate is a contributory cause of the fall of the salivary $Na^+:K^+$ ratio during Na^+ depletion, but this factor accounts for only 25–50 % of the fall (Denton, 1956, 1957*a*).

(iii) An increase of the plasma Na^+ concentration by 4–40 m-equiv/l. in the parotid arterial field of a Na^+-depleted sheep, produced by slow intracarotid infusion of a concentrated NaCl solution, does not cause any significant immediate change in the salivary $Na^+;K^+$ ratio. However, if the amount of Na^+ injected is sufficient to cause a significant change in the sheep's Na^+ balance, the parotid salivary $Na^+:K^+$ ratio rises rapidly after a latent period of 1–2 hr. The latter effect may be reproduced by rapid bilateral adrenalectomy in a Na^+-depleted sheep, suggesting that the response is due to inhibition of electrolyte-active adrenal secretion (McDonald & Denton, 1956; Denton

& McDonald, 1956, 1957). The fact that the response to rapid change of Na^+ balance occurs during constant secretomotor stimulation by acetylcholine supports the view that the stimulus to the parotid is humoral, and unpublished work (McDonald & Raschke) shows that parasympathetic denervation of the parotid gland does not prevent the normal response to Na^+ deficit and the rapid correction of it.

(iv) The injection of DOCA (deoxycorticosterone acetate) in normal and adrenalectomized sheep causes a fall in the salivary $Na^+:K^+$ ratio (Goding & Denton, 1956). The normal parotid response to Na^+ depletion does not occur in an adrenally insufficient sheep (Goding & Denton, 1956, 1957), but the response can be imitated by provision of a gradually increasing amount of cortical hormone during Na^+ depletion (Denton, Goding & McDonald, unpublished). If an adrenalectomized sheep with a parotid fistula is given an increased but constant amount of DOCA each day (e.g. 20 mg), the parotid salivary $Na^+:K^+$ ratio falls, and an equilibrium state occurs at a ratio of 2·0–3·0. If the Na^+ supplement of the sheep is then withdrawn, the resulting Na^+ deficit causes the salivary $Na^+:K^+$ ratio to decrease further to 0·3–0·5 (Goding & Denton, 1956). Experimental analysis of this second fall of the salivary $Na^+:K^+$ ratio indicates that it is largely attributable to a decrease of secretion rate, and is reversed by intracarotid ACh infusion (Denton, Goding & McDonald, unpublished). The presumption, on this evidence, is that the increased electrolyte-active adrenal secretion, and the local parotid secretion rate factor are jointly sufficient conditions to cause the fall in the salivary $Na^+:K^+$ ratio during Na^+ deficit in a normal sheep.

This evidence suggests the hypothesis that there is a receptor tissue in the body of the animal which responds to some consequence of change in Na^+ balance by altering the electrolyte-active secretion of the adrenal gland. The crucial questions are *the locus of the sensitive tissue, and the precise nature of the stimulus to it.*

This paper deals with the changes which occur in the extracellular fluid as a result of Na^+ depletion, and with experimental procedures designed to cause variation of the individual extracellular components with the object of identifying the stimulus to the hypothetical receptor determining the parotid response during Na^+ depletion. The principal result is that the salivary $Na^+:K^+$ ratio falls in response to a Na^+ deficit, even though concurrent water depletion causes the plasma Na^+ concentration to rise above the control period concentration. Restoration of free water intake during the Na^+ deficit period has been found to have no influence on the course of the salivary $Na^+:K^+$ ratio, though there occurs a rapid and large change of the plasma Na^+ concentration, and of other components of the *milieu intérieur*. Reasons are advanced (see Discussion) for proposing that this finding does not necessarily exclude the possibility that the receptor tissue determining the parotid response reacts to change of the Na^+ concentration of the plasma. Other experiments involving variation in ionic intake during Na^+ depletion suggest that the parotid response during Na^+ depletion is not caused by a change in whole blood pH, plasma K^+, Cl^- or HCO_3^- concentrations or by a decrease of the plasma $Na^+:K^+$ ratio. The normal form of the parotid response to Na^+ depletion of water-deficient and water-loaded sheep and thyroidectomized sheep provides evidence against the neurohypophysis or the thyroid gland playing a role in this mechanism in the normal animal.

METHODS

Cross-bred Merino sheep were used. P.F. 1, P.F. 18 and P.F. 21 had Pavlov-Glinski parotid fistulae, P.F. 13, P.F. 16, P.F. 17, P.F. 33 and P.F. 40 had Wright parotid fistulae (Denton, 1957*b*). With the exception of P.F. 18, each sheep had a carotid loop. Arterial blood samples were taken from the carotid loop without disturbance of the animal. In the experiments involving assessment of change of plasma K^+ concentrations it was important to analyse arterial and not jugular venous blood, since it has been shown (Blackwood & Wishart, 1936; Coats, Denton & Wright, unpublished) that the electrolyte composition of the latter may be significantly changed by the activity of the parotid gland. The conduct of total electrolyte balance studies in stainless steel metabolism cages, the method of production of Na^+ depletion, and the technique of intra-carotid infusion of ACh have been described elsewhere (Denton, 1956, 1957*a*).

The chemical methods employed have been described previously (Denton, Wynn, McDonald & Simon, 1951; Denton, 1956).

RESULTS

The effect of water depletion on the fall of parotid salivary $Na^+:K^+$ *ratio during* Na^+ *depletion*

In these experiments the water intake of a sheep was restricted during Na^+ depletion. This caused a rise of plasma Na^+ concentration during Na^+ depletion, instead of the usual fall.

Two experiments on P.F. 1 permit comparison of a standard episode of Na^+ depletion with one in which the water intake was severely restricted for the first 7 days of the Na^+ depletion period. In both experiments the sheep had free access to water during the control period and a similar diet which did not vary significantly in electrolyte composition. During each control period the sheep was given 595 m-equiv of $NaHCO_3$ each day by rumen tube, and in both instances a supplement of 200 m-equiv of $KHCO_3$ was given by rumen tube throughout the whole experiment. This was to ensure that there was an ample supply of K^+ available to meet the increase of parotid K^+ output during Na^+ depletion. Thus no question would arise of complicating physiological side effects due to the parotid gland causing a deficit of body K^+. It has already been shown (Denton, 1956) that provision of this supplement does not significantly change the normal course of parotid response to Na^+ depletion. In both instances total balance studies showed that the sheep was in Na^+, K^+ and Cl^- equilibrium during the control period. The supplementary

Na^+ intake was withdrawn for 14 days in the standard episode (○) and for 12 days in the episode which involved water depletion (×) (Figs. 1–3). In the standard episode the average intake of water during the control period was 4·8 l./day and during the 14 days of Na^+ depletion it was 4·2 l./day. Fig. 3 shows that, for the first 7 days of the experiment under consideration, water

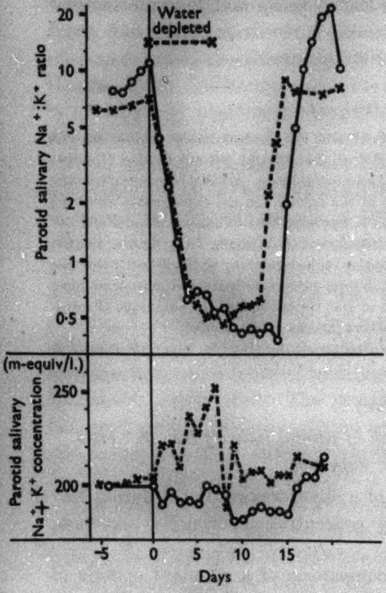

Fig. 1

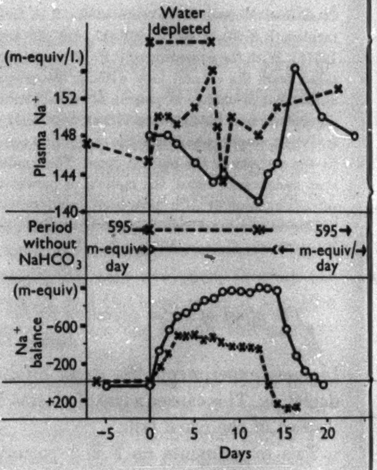

Fig. 2

Fig. 1. P.F. 1 (1954). Comparison of the effect on parotid salivary $Na^+:K^+$ ratio and parotid salivary osmotic pressure of a standard episode of Na^+ depletion (○) with one in which the water intake was severely restricted for the first 7 days of Na^+ depletion (×). Na^+ depletion commenced at day 0 in both instances, and was produced by withholding the daily Na^+ supplement of 595 m-equiv $NaHCO_3$.

Fig. 2. P.F. 1 (1954). The effect on plasma Na^+ concentration and the cumulative Na^+ balance of the procedures described in Fig. 1. The daily Na^+ supplement was withheld for 14 days in the standard episode and for 12 days in the episode which involved 7 days of water depletion. Conventions as for Fig. 1.

intake was reduced to one-third to one-sixth of that in the corresponding control period. On the morning of the 7th day the animal was permitted free access to water, and drank 3·5 l. during the first 5 min.

The principal finding of the experiment was that the plasma Na^+ concentration rose during the water depletion episode (Fig. 2), yet the parotid salivary

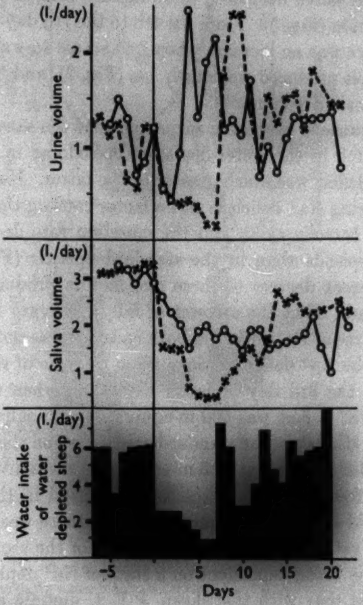

Fig. 3. P.F. 1 (1954). The effect on the daily volume of urine and saliva of the procedures described in Fig. 1. The water intake during the episode in which the animal was water depleted during the first 7 days of Na⁺ depletion is also shown. In the standard episode the average intake during Na⁺ deficit was 4·2 l./day.

Na⁺:K⁺ ratio (Fig. 1) fell at the same rate as in the standard episode in which the usual fall in plasma Na⁺ occurred. The resumption of free access to water on the 7th day caused a very large fall of plasma Na⁺ concentration, but this had little influence on the course of the parotid salivary Na⁺:K⁺ ratio. Other features of the water depletion experiment were:

(1) The sheep lost 5 kg in weight in 7 days, whereas in the standard episode it lost 2·3 kg in the first 8 days.

(2) The salivary osmotic pressure rose (Fig. 1) and, like the plasma Na⁺ concentration, fell sharply with resumption of free intake of water.

(3) The urine volume decreased to less than 0·2 l./day (Fig. 3). There was a large diuresis with resumption of free intake of water. In the standard episode there was a diuresis between the 4th and 7th days. This was probably a consequence of osmoregulation in the face of a declining plasma Na⁺ concentration.

(4) The volume of saliva decreased more rapidly than in a standard episode of Na^+ depletion (Fig. 3). From the 4th to the 7th day it was 0·66–0·45 l./day, but there was an increase when free access to water was allowed.

(5) The Na^+ deficit produced was much less (Fig. 2), and this was due to the rapid fall of saliva volume.

In the first instance it may seem surprising that the salivary $Na^+:K^+$ ratio fell almost as much in the water-depletion episode as in the standard one, though the Na^+ deficit was much greater in the latter. However, decrease of secretion rate during Na^+ deficit is also a factor causing the salivary $Na^+:K^+$ ratio to fall (see Introduction), and the secretion rate declined more in the water-depletion episode than in the standard episode (Fig. 3). Hence the operation of different degrees of these two factors produced approximately the same end result. That the amount of fall of salivary $Na^+:K^+$ ratio was related to the degree of Na^+ deficit in both episodes was shown by comparison of the ratio and the Na^+ deficit on days when the rate of secretion coincided. For example, on the 3rd day of water depletion, when secretion rate was 1·46 l./day, the Na^+ deficit was 500 m-equiv, and the salivary $Na^+:K^+$ ratio was 1·38. On the 4th day of the standard episode, when the secretion rate was 1·47 l./day, the Na^+ deficit was 725 m-equiv and the salivary $Na^+:K^+$ ratio was 0·63. When free access to water was permitted in the water depletion episode the parotid secretory rate increased, and there was a small rise in the salivary $Na^+:K^+$ ratio. In relation to the effect of the secretion rate on salivary $Na^+:K^+$ ratio being dependent on a state of Na^+ depletion for its action, it may be recalled that with this sheep, P.F. 1, dietetic experiments established that a fall of secretion rate from 3·17 to 1·47 l./day had no effect on the salivary $Na^+:K^+$ ratio while the animal was in normal Na^+ balance (Denton, 1956). The greater decrease of secretion rate in water depletion may have been due to greater systemic circulatory effects, and also to the diminished food intake and thus diminished ruminal activity.

Combined water and Na^+ depletion experiments were made on other sheep under differing conditions. With P.F. 21 (Fig. 4) water intake was reduced first, and reduction of urine and saliva volume and increase of salivary osmotic pressure indicated that significant water deficit preceded Na^+ depletion. When Na^+ intake was reduced below the animal's Na^+ output, the salivary $Na^+:K^+$ ratio fell, though the arterial plasma Na^+ concentration rose as high as 165 m-equiv/l. Free access to water caused the plasma Na^+ concentration to fall by 27 m-equiv/l. but this appeared to have little effect on the course of the salivary $Na^+:K^+$ ratio (Fig. 4). With P.F. 16, at the end of a control period, the salivary $Na^+:K^+$ ratio was 30, and the plasma Na^+ concentration was 152 m-equiv/l. Free access to water was permitted for the first 3 days of withdrawal of Na^+ supplement. On the 3rd day the salivary $Na^+:K^+$ ratio was 2·0. No water was given to the sheep between the 3rd and the 6th day of

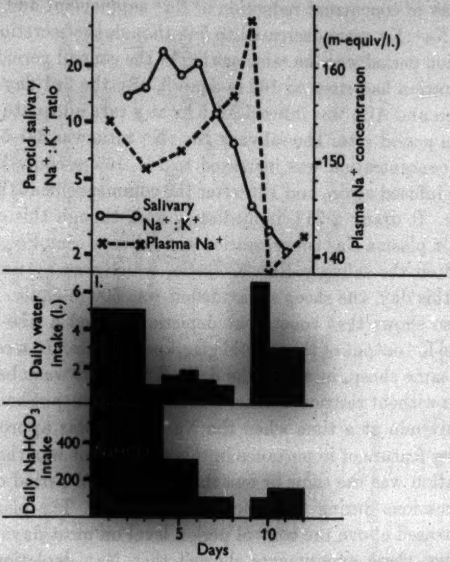

Fig. 4. P.F. 16 (1955). The effect on plasma Na^+ concentration and parotid salivary $Na^+:K^+$ ratio of restriction of the daily water intake before and during reduction of the amount of the daily Na^+ supplement.

Na^+ depletion, and between the 6th and 7th day only 1·0 l. was given. On the 5th, 6th and 7th days the plasma Na^+ concentration was found to have risen to 156–157 m-equiv/l. and the parotid salivary $Na^+:K^+$ ratio to have fallen to 0·85–0·25. The changes in salivary osmotic pressure were similar to those shown in Fig. 1. Another experiment on P.F. 16, conducted similarly to that on P.F. 1 (Figs. 1–3), gave a similar result.

The fall in salivary $Na^+:K^+$ ratio during Na^+ depletion is dependent on two principal factors, the increased secretion of the adrenal cortex, and a decrease of salivary secretion rate in the presence of Na^+ depletion (see Introduction). In order to establish formally the crucial fact that the fall in the salivary $Na^+:K^+$ ratio during concurrent water and Na^+ depletion did involve an increased output of electrolyte-active adrenal steroid, the following experiment was made. On succeeding days of concurrent depletion the salivary $Na^+:K^+$ ratio was observed for comparable periods under conditions where an ipsilateral intracarotid infusion of ACh (Denton, 1957*a*) ensured that the parotid secretion rate was as great as during the control period, when there was a normal $Na^+:K^+$ ratio. The results (P.F. 40, Table 1) support such a conclusion. By the second day of concurrent reduction of Na^+ supplement and water intake, the salivary $Na^+:K^+$ ratio decreased to 5·0, though the secretion rate during the observation period was the same as that of the control period. The plasma Na^+ concentration had risen to 162 m-equiv/l. By the 3rd day the secretion rate had fallen and ACh was infused for 5 hr at a rate adequate to increase it to the control period rate. The salivary $Na^+:K^+$ ratio was 3·0–5·0 though the plasma Na^+ concentration was increased to 161–165 m-equiv/l. On the 4th day ACh was infused again, and 1 hr after the commencement the animal was offered water. It drank 2·76 l. immediately and, though this caused a considerable fall in plasma Na^+ concentration and salivary osmotic pressure, there was no effect on the salivary $Na^+:K^+$ ratio, which was by now reduced to 2·6–2·9. On this day, the sheep's Na^+ deficit was 500 m-equiv.

Table 1 also shows that concurrent depletion caused a five- to sevenfold increase of the K^+ output of the parotid gland per minute. In a control experiment on the same sheep, in which the daily K^+ output was observed during Na^+ depletion without restricted water intake, the K^+ output had increased to 100–150 μequiv/min at a time when the Na^+ deficit was approximately the same. Another feature of importance in this experiment was that the plasma K^+ concentration was the same or less than the control period concentration on several occasions during the concurrent depletion. The plasma $Na^+:K^+$ ratio was increased above the control period level on most days.

Summarizing, these experiments showed that Na^+ depletion caused the parotid salivary $Na^+:K^+$ ratio to fall, even though concurrent water depletion caused the plasma Na^+ concentration to rise relative to the control period. The large rapid fall of plasma Na^+ concentration which occurred when free access to water was permitted during Na^+ depletion had little influence on the course of the salivary $Na^+:K^+$ ratio. Experiments in which ACh was infused in order to eliminate the influence of decreased secretion rate on the salivary $Na^+:K^+$ ratio supported the proposition that the fall of salivary $Na^+:K^+$ during concurrent water and Na^+ depletion resulted from an increased secretion of electrolyte-active adrenal steroid.

The effect of greatly reduced dietary intake of K^+ *and* Cl^- *on the parotid response to* Na^+ *depletion*

The plasma K^+ *concentration.* The mean K^+ concentration of twenty-five specimens of arterial blood taken from P.F. 13, P.F. 16 and P.F. 33, whilst in normal Na^+ balance was 4·4 ± 0·4 (S.D.) m-equiv/l. During Na^+ depletion the plasma K^+ concentration rises. Eleven specimens taken from these sheep when Na^+-depleted had a K^+ concentration of 5·6 ± 0·8 (S.D.) m-equiv/l. With the aim of seeing whether the parotid response to Na^+ depletion would occur if the plasma K^+ concentration were made to decrease, P.F. 13 was given an artificial diet. This consisted of a mixture of 0·1 kg starch, 0·1 kg

TABLE 1. An experiment on P.F. 40 (May 1957) in which Na^+ depletion and water depletion were produced concurrently. The effect on plasma Na^+ and K^+ concentrations and salivary $Na^+:K^+$ ratio, osmotic pressure, and K^+ output were compared on succeeding days of depletion under conditions where secretion rate was raised to the control period rate by ipsilateral intracarotid ACh infusion. The effect of permitting free access to water was observed on the 4th day of depletion

		Parotid saliva						Plasma						
Day	Time	Volume (ml.)	Rate (ml./min)	$Na^+:K^+$	$Na^+ + K^+$ (m-equiv/l.)	K^+ output (μequiv/min)	Whole blood pH	Na^+ (m-equiv/l.)	K^+ (m-equiv/l.)	$Na^+:K^+$	Water intake during preceding 24 hr (l.)	Supplementary Na^+ intake (m-equiv/day)	Urine (l./day)	Saliva (l./day)
−1	—	—	—	—	—	—	—	—	—	—	Not restricted	595	0·24	3·38
0	11.28–12.28	72	1·20	23·0	192	10	7·31	144	4·4	32·7	Not restricted	298	1·05	3·04
1	10.04–11.04	117	1·95	25·6	186	13	7·29	155	5·1	30·4	1·0	100	1·24	2·21
2	10.02–11.02	33	—	5·0	205	—	7·31	162	4·4	36·8	1·0	—	0·13	1·34
	14.34–15.34	75	1·25	6·7	200	33	—	—	—	—	—	—	—	—
3	10.03–11.03	12	0·20	3·9	220	9	7·23	165	4·7	35·1	nil	—	0·05	0·74
ACh infusion 2×10^{-5} at 0·4 ml./min	12.29–12.59	42	1·40	5·0	260	60	—	—	—	—	—	—	—	—
	12.59–14.29	95	1·06	5·3	250	42	—	—	—	—	—	—	—	—
	14.29–15.29	92	1·53	4·8	253	66	—	—	—	—	—	—	—	—
	15.29–16.10	72	1·76	4·0	217	76	—	—	—	—	—	—	—	—
	16.34–17.34	67	1·12	3·1	216	63	—	—	—	—	—	—	—	—
	16.46	—	—	—	—	—	—	161	4·1	39·3	—	—	—	—
4	10.35	—	—	—	—	—	7·20	153	4·8	31·9	0·15	—	nil	0·24
ACh infusion 2×10^{-5} at 0·4 ml./min	10.42–11.00	20	1·11	2·6	227	69	—	—	—	—	—	—	—	—
	11.00–11.20	25	1·25	2·7	224	76	—	—	—	—	—	—	—	—
	11.21	Offered water; drank 2·76 l. in 4 min; water then removed												
	11.29–11.44	25	1·67	2·7	179	82	—	—	—	—	—	—	—	—
	11.49–12.09	25	1·25	2·7	191	65	—	—	—	—	—	—	—	—
	12.15–12.45	28	—	2·7	186	—	—	—	—	—	—	—	—	—
	12.29	—	—	—	—	—	—	144	3·5	41·1	—	—	—	—
	13.20–13.50	42	1·40	2·6	186	71	—	—	—	—	—	—	—	—
	13.52	—	—	—	—	—	—	142	3·5	40·6	—	—	—	—
	13.59–14.29	38	1·27	2·9	183	60	—	—	—	—	—	—	—	—
	14.32	Offered water, drank 1·10 l. in 2 min.												

casein, 0·2 kg sucrose, 0·1 kg filter-paper pulp, and 0·2 kg lucerne chaff each day, moistened with 0·5 l. water. Adequate amounts of vitamins A, D and B group were added. The diet provided a daily intake of 8 m-equiv of Na^+, 107 m-equiv of K^+, and 34 m-equiv of Cl^-. Provided there was no Na^+ deficit, the animal could be maintained on this diet for 1–2 months. The K^+ intake was less than the maximum parotid K^+ output observed under conditions of Na^+ depletion (Denton, Goding & McDonald, unpublished). Because

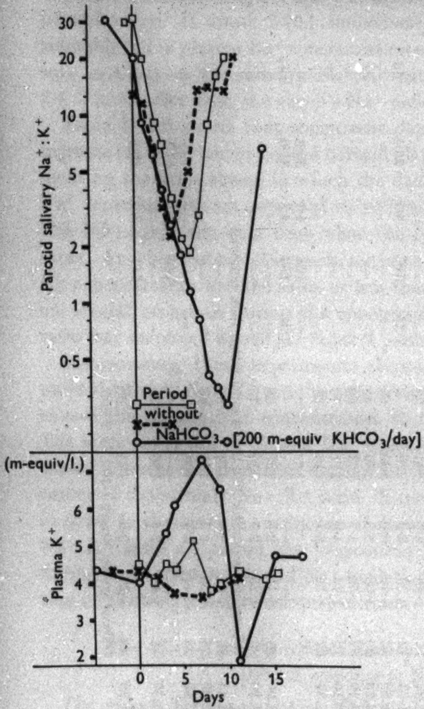

Fig. 5

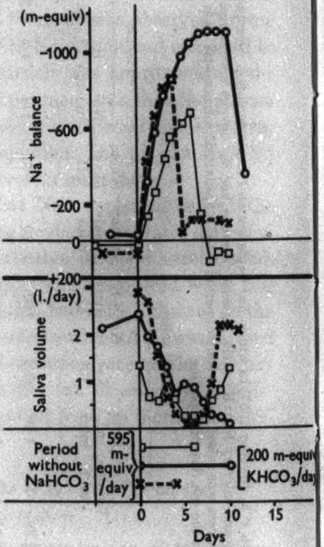

Fig. 6

Fig. 5. P.F. 13 (1955). The effect on parotid salivary $Na^+:K^+$ ratio and the plasma K^+ concentration of experiments in which Na^+ depletion was produced when the animal received an artificial diet with a much reduced K^+ content. Two such episodes (×) and (□) are compared with a control episode (○) in which the sheep was given 200 m-equiv of $KHCO_3$/day.

Fig. 6. P.F. 13 (1955). The effect on the cumulative Na^+ balance and the saliva volume of the experimental procedures described in Fig. 5. The period in each instance during which the daily Na^+ supplement was withheld is also shown. Conventions as in Fig. 5.

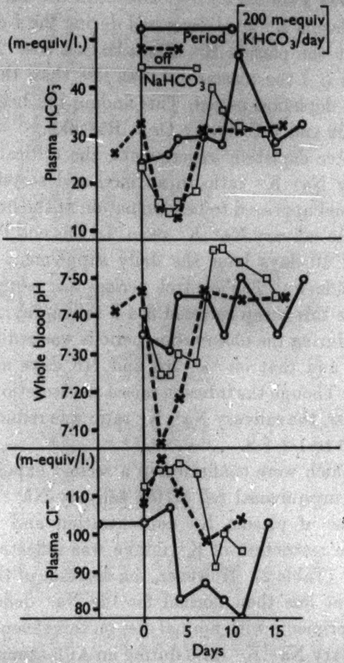

Fig. 7. P.F. 13 (1955). The effect on the whole blood pH, the plasma HCO$_3^-$, and the plasma Cl$^-$ of the occurrence of Na$^+$ depletion under the conditions described in Fig. 5.

of the casein component, the HPO$_4^{2-}$ intake (approximately 100 m-equiv/day) was adequate to cover the salivary loss. The effect, under these conditions, of withdrawal of the daily supplement of 595 m-equiv of NaHCO$_3$ was compared on two occasions with the findings in a control experiment in which the animal was given also a daily supplement of 200 m-equiv of KHCO$_3$ throughout the experiment (Figs. 5, 6, 7).

It was found that Na$^+$ depletion in the absence of an adequate K$^+$ intake had a severe impact on the sheep's condition, and the experiment could not be continued longer than 4–6 days. The saliva volume (Fig. 6) decreased even more precipitately than during water depletion, and after 2–4 days the sheep ate very little. The principal finding was that the parotid salivary Na$^+$:K$^+$ ratio fell though the plasma K$^+$ concentration decreased. Fig. 6 shows clearly that in one experiment (×) during 4 days the progression of the Na$^+$ deficit closely paralleled the control with adequate K$^+$ intake. Comparison with Fig. 5 shows a similar correspondence in the fall of salivary $Na^+:K^+$ ratio though the plasma K^+ level decreased during the 4 days, whereas in the control experiment the plasma K^+ concentration rose 2 m-equiv/l. In the second experiment (□) the plasma K^+ was less than the control value on 2 days of the Na^+ depletion period. This finding has been confirmed in two other sheep (P.F. 39 and P.F. 40) by Dr J. Raschke.

As with the water depletion experiments, the critical point in assigning the fall in salivary $Na^+:K^+$ ratio to an increased secretion of electrolyte-active adrenal steroid appeared to be elimination of the influence of decreased secretion rate on the salivary $Na^+:K^+$ ratio. To this end P.F. 33 was given an artificial diet, and 10 days later the daily supplement of 830 m-equiv of $NaHCO_3$ was withdrawn. The control period Na^+ supplement was larger than usual because this sheep secreted 3–4 l. saliva/day. Table 2 shows that the secretion rate during the observation periods was reduced by the 2nd day of Na^+ depletion, and that on the 3rd and 4th days an intracarotid ACh infusion was given. Though the infusion raised the secretion rate to the control period rate or higher, the salivary $Na^+:K^+$ ratio was reduced from the control period level of 28·6 to 1·2–3·8.

These results, which were confirmed in a second experiment on the same sheep, showed an unequivocal fall of the salivary $Na^+:K^+$ ratio, despite a concurrent decrease of plasma K^+ concentration and rise of the *plasma* $Na^+:K^+$ ratio. The restriction of K^+ intake was reflected in active urinary conservation of K^+ (Table 2). However, the decrease of the salivary $Na^+:K^+$ ratio was somewhat less than normal for the Na^+ deficit. In three other Na^+-depletion experiments with normal diet on this sheep (Denton & McDonald, 1957) the salivary $Na^+:K^+$ ratio during an ACh-stimulated secretion rate of 2·0–3·0 ml./min was 1·64–2·51 when the Na^+ deficit ranged between 644 and 690 m-equiv. Here, on the 3rd day, the Na^+ deficit was 648 m-equiv (Table 2) and the salivary $Na^+:K^+$ ratio was 3·0–3·8 when saliva flow rate was stimulated to 2·0–4·0 ml./min by ACh infusion. In the second experiment on P.F. 33, referred to above, the Na^+ deficit on the 4th day was 580 m-equiv, and the salivary $Na^+:K^+$ was 2·5–3·1 under conditions of ACh-stimulated salivary flow rate of 1·3–2·0 ml./min. This slight limitation of parotid response to Na^+ deficit under these conditions of K^+ restriction would not appear to put in jeopardy the main fact of its occurrence during lowered plasma K^+ concentration. Evidence will be presented in another communication suggesting that the explanation of the limitation lies in restriction of supply of K^+ to the parotid gland rather than in diminished output of adrenal steroid.

The plasma $Na^+:K$ *ratio.* As would follow from the usual behaviour of the plasma Na^+ and K^+ concentrations, the plasma $Na^+:K^+$ ratio falls in Na^+ depletion. However, study of the results of the low K^+ diet experiments on P.F. 13, P.F. 33 (Table 2), P.F. 39 and P.F. 40 showed that on twenty-one

TABLE 2. P.F. 33 (June, 1957). An experiment in which dietary K^+ intake was restricted during Na^+ depletion. The effect on plasma Na^+ and K^+ concentrations, the salivary $Na^+:K^+$ ratio and K^+ output, and the daily urinary K^+ excretion were compared on succeeding days of Na^+ depletion. On the 3rd and 4th day of Na^+ deficit there was a large decrease of the daily saliva secretion rate, and on these days the salivary $Na^+:K^+$ ratio was observed during intracarotid ACh stimulation adequate to raise the secretion rate to the control period rate or higher

		Parotid saliva				Plasma							
Day	Time	Volume (ml.)	Rate (ml./min)	$Na^+:K^+$	K^+ output (μequiv/min)	Whole blood pH	Na^+ (m-equiv/l.)	K^+ (m-equiv/l.)	$Na^+:K^+$	Supplementary Na^+ intake (m-equiv)	Cumulative Na^+ balance (m-equiv)	Urine K^+ excretion (m-equiv/day)	Saliva (l./day)
−1	—	—	—	—	—	—	—	—	—	833	—	83	—
0	09.56–10.56	76	1·3	28·6	7	7·40	141	4·7	30·0	—	+ 40	77	3·21
	15.46–16.46	156	2·6	13·4	30	7·28	150	4·7	31·9	—	—	—	—
1	10.17–11.17	114	1·9	4·5	55	7·26	136	3·6	37·8	—	−400	52	3·01
	15.47–16.47	86	1·4	2·9	53	7·20	125	3·4	36·8	—	—	—	—
2	09.33–10.33	27	0·45	0·7	41	7·09	131	3·5	37·4	—	−590	4	1·75
	15.37–16.37	27	0·45	0·7	39	7·14	125	3·1	40·3	—	—	—	—
3	09.25–10.25	15	0·3	0·2	37	7·07	125	3·2	39·1	—	−648	2	0·79
ACh infusion 5×10^{-6} at 0·05–0·2 ml./min	14.19–14.25	24	4·0	3·8	128	—	—	—	—	—	—	—	—
	14.26–14.28	24	2·0	3·0	76	—	—	—	—	—	—	—	—
	14.40–14.57	7	0·4	1·0	28	—	—	—	—	—	—	—	—
	14.58–15.12	52	3·7	3·6	122	—	—	—	—	—	—	—	—
	15.13–15.25	39	3·3	3·7	116	—	—	—	—	—	—	—	—
4	10.19–11.01	9	0·2	0·1	28	7·06	128	3·9	32·8	—	−670	2	0·54
ACh infusion 5×10^{-6} at 0·05–0·2 ml./min	11.41–11.50	28	3·1	1·9	164	—	—	—	—	—	—	—	—
	11.52–12.03	9	0·8	0·9	62	—	—	—	—	—	—	—	—
	12.09–12.21	21	1·7	1·2	124	—	—	—	—	—	—	—	—
	12.22–12.40	52	2·9	1·9	154	7·03	123	3·0	41·0	—	—	—	—
	16.46–17.34	—	—	—	—	7·08	125	3·4	36·8	—	—	—	—
		—	—	—	—	7·05	120	3·2	37·5	—	—	—	—

occasions during Na^+ deficit, the plasma $Na^+:K^+$ ratio was greater than in the corresponding control period, yet, as indicated, the salivary $Na^+:K^+$ ratio fell. Consideration of the water depletion experiments from this viewpoint (Table 1) also showed that on some days the plasma $Na^+:K^+$ ratio was higher than during the control period. This was because of both a rise in plasma Na^+ and a fall of plasma K^+ relative to the control period concentrations.

The whole blood pH and plasma HCO_3^- *and* Cl^- *concentrations.* The observations made in the course of the experiments on P.F. 13 (Fig. 7) indicated that the fall of the salivary $Na^+:K^+$ ratio during Na^+ depletion was largely independent of the changes in whole blood pH, plasma HCO_3^- and plasma Cl^- concentrations. The decrease of pH and plasma HCO_3^- was very great in the experiments in which the K^+ intake was low. However, on some days of the experiment in which adequate K^+ intake was given, the pH and HCO_3^- were slightly higher than during the control period of adequate daily Na^+ supplement. The difference between the experiments lies in the much more efficient renal behaviour in this latter instance. For simple quantitative reasons set out in detail elsewhere (Denton *et al.* 1951; Denton, 1957*b*), the survival of an animal which loses from the body large volumes of fluid containing Na^+ in excess of Cl^- depends to a great extent on complete renal conservation of Na^+ along with *an absolute increase in the* Cl^- *excretion by the kidney*. Though this renal behaviour occurred in all the experiments, it was much more efficient in the experiment in which an adequate K^+ intake was provided, and there caused a negative Cl^- balance of 344 m-equiv during the Na^+ depletion period. That is, the kidney removed a significant proportion of the extracellular Cl^- in response to the severe stress on the normal extracellular electrolyte relationships caused by the loss of 1190 m-equiv of Na^+ and only 71 m-equiv of Cl^- from the parotid fistula. This more efficient renal behaviour is reflected in the plasma Cl^- graph (Fig. 7) which shows a fall of plasma Cl^- in contrast to the other two experiments in which it was raised above control period level during most of the Na^+ depletion period.

The effect of a large increase in intake of Cl^- *and* HPO_4^{2-} *during* Na^+ *depletion*

Three experiments were made on P.F. 1 under conditions of normal diet in which the sheep was given a supplement of 100 m-equiv of KCl/day throughout the experiment. This, along with the diet, gave a total Cl^- intake of 280 m-equiv/day. In two of the experiments the phosphate intake was increased by a supplement of 100 mM-K_2HPO_4/day. In the three instances the fall of the salivary $Na^+:K^+$ ratio upon Na^+ withdrawal followed very closely the course of the standard episode shown in Fig. 1.

Summarizing, this group of experiments showed that a rise of K^+ concentration was not an essential condition for the parotid response to Na^+

depletion. Nor did it appear that a fall of the plasma $Na^+:K^+$ ratio was essential. The parotid response followed the usual course whether the whole blood pH, plasma HCO_3^- and plasma Cl^- fell or rose. A large decrease or increase of Cl^- intake and a large increase of HPO_4^{2-} intake had no conspicuous influence on the parotid mechanism.

The neurohypophysis and the thyroid gland

For comparison with the water depletion experiments, P.F. 1 was given a large daily water load during the control and Na^+-depletion period. Each morning and afternoon 2 l. of water was administered by rumen tube. This succeeded in producing a urine flow of 1·5–2·5 l./day during the depletion period. This procedure caused no deviation from the usual course of the salivary $Na^+:K^+$ ratio during Na^+ depletion. Consideration of the effect on urine volume of the water-depletion and water-loading experiments in the light of the physiological events involved in water diuresis (Verney, 1947) suggests that the parotid response to Na^+ depletion is not affected by the concentration of antidiuretic hormone in the blood.

The thyroid gland of P.F. 18 was removed (May, 1955), and during the following 6 months the animal was submitted to several episodes of Na^+ depletion. The parotid response was normal, but the renal conservation of Na^+ did not appear to be as efficient as in a normal animal.

DISCUSSION

The experiments described in the Introduction indicate that the major factor in the parotid response to Na^+ depletion is the elaboration of an electrolyte-active secretion by the adrenal cortex. Putting aside here the question of the locus of the tissue sensitive to change of the animal's Na^+ balance, it seemed a reasonable working hypothesis that the stimulus to this receptor was humoral, and was a consequence of some effect of the negative Na^+ balance upon the *milieu intérieur*. A logical first consideration was the plasma Na^+ concentration since, consistently, there is a fall during Na^+ depletion. On the other hand, examination of the plasma Na^+ concentration in a large series of experiments in one animal does not suggest a simple relation between the amount of the fall of Na^+ concentration and the extent of salivary $Na^+:K^+$ change. The change of Na^+ concentration during Na^+ depletion would not be dependent simply on the degree of Na^+ deficit, but would depend also on the activity of the osmoregulating mechanism and the possibility of a varying degree of accommodation in the osmoreceptors (Verney, 1947). Against this is the close correspondence (60–120 min delay) between rise of the plasma Na^+ concentration and rise of the salivary $Na^+:K^+$ ratio in experiments in which a Na^+ deficit is rapidly corrected by a large intraruminal dose of Na^+ (McDonald, unpublished). The results set out in this paper show decisively that the parotid salivary $Na^+:K^+$ ratio may fall in response to a Na^+ deficit even though concurrent water depletion causes the plasma Na^+ to rise above the control period concentration. However, it would not seem that this necessarily eliminates the fall of plasma Na^+ concentration during a standard episode of Na^+ depletion as the specific stimulus to the receptor tissue controlling the output of electrolyte-active steroid. It is quite possible that the stimulus to the receptor tissue involves a change of concentration gradient between an extracellular and intracellular component with resultant change in the intracellular component. Water depletion would cause an equivalent change in both the intracellular and extracellular components. The superimposed Na^+ depletion could still distort the relationship as in the normal animal. Another explanation might reside in the hypothetical receptor being responsive to a change in the load of Na^+ presented to it per unit time. This would reconcile the similar findings in the apparently divergent situations of water depletion and the standard Na^+ deficit since haemodynamic deterioration, with consequent decrease of Na^+ load, would be common to both episodes.

In relation to the experiments on Na^+ depletion with the low K^+ diet, it should be emphasized that this restriction seriously disrupted the animal's ability to withstand Na^+ depletion. The changes in plasma constituents were more precipitate. None the less, the experiments have shown that the parotid response may be dissociated from the direction of change of plasma K^+ concentration and plasma $Na^+:K^+$ ratio, and this conclusion is also supported by some of the results in the water depletion experiments. The finding, in the experiments involving comparison of high and low K^+ intakes whilst on artificial diet, of a lack of correspondence between the parotid response and the whole blood pH, plasma HCO_3^- and Cl^- concentrations, is substantiated by the examination of plasma findings in fourteen other total balance studies of Na^+ depletion made with sheep on normal diet. Often there was a large fall of pH and plasma HCO_3^- and a rise of plasma Cl^- during the first 3 days, followed by some restitution of pH and plasma HCO_3^- and a fall of plasma Cl^- below control period level during the subsequent days of Na^+ depletion, whereas the salivary $Na^+:K^+$ ratio continued to fall parallel with the Na^+ deficit (Denton, 1957*b*).

An assertion that the results of these experiments are of physiological importance turns upon one critical point. This is the use of the salivary $Na^+:K^+$ ratio as a functional index of the electrolyte-active secretion of the adrenal cortex. If this be valid, it then follows that Na^+ depletion will provoke an increased secretion of electrolyte-active adrenal steroid despite the direction of the changes in the *milieu intérieur* contrived by the experimental conditions set out in this paper. Objection to this assertion might be made along the following lines. Whereas, in a sheep in normal Na^+ balance, a considerable decrease of secretion rate has little effect on the salivary $Na^+:K^+$ ratio

(Denton, 1956), in a Na^+-depleted sheep the relation between secretion rate and the salivary $Na^+:K^+$ is linear; a decrease of secretion rate causing a fall of the ratio. It could be argued that, in both the water-depletion and low K^+ diet experiments, Na^+ depletion caused a very large fall of secretion rate (Figs. 3 and 5, Tables 1 and 2), and this alone was responsible for the decrease of the salivary $Na^+:K^+$ ratio. Against this is the fact that, if the daily Na^+ supplement be withdrawn from an adrenalectomized sheep with a parotid fistula *without* increasing the DOCA dosage above maintenance level, there is little salivary response despite a precipitate decrease of secretion rate. That is, an increased adrenal secretion appears necessary for the occurrence of this parotid response to Na^+ depletion (Denton, Goding & McDonald, unpublished). In the experiments recorded in this paper the objection would seem to have been formally eliminated by making numerous observations of the salivary ratio during Na^+ deficit under conditions where the secretion rate was stimulated to a level as great as or greater than during the control period.

The results in this paper emphasize the difficulty of obtaining specific answers to the two questions raised in the Introduction. Consideration of this in the light of the finding by Denton & McDonald (1957) that the parotid response to rapid correction of Na^+ deficit *cannot be elicited in an anaesthetized animal* provoked the view that a solution might be forthcoming only with the development of a method of isolating the arterial supply of the adrenal gland in a conscious undisturbed animal. If it be that the adrenal itself is the locus of the sensitive tissue, this would permit a precise local alteration of the electrolyte composition of the blood perfusing the adrenal gland to be imposed without significant disturbance of the electrolyte balance of the animal. This end has been attained in our laboratory (Goding, McDonald & Wright, 1957; Goding, 1957) by transplantation of the left adrenal gland on to a carotid artery–jugular vein skin loop in the neck. At a subsequent operation the right adrenal gland is removed. The inflation of a pneumatic cuff applied to the loop cranial to the transplant results in the carotid artery supplying only the adrenal gland, and a needle attached to a motor-driven syringe can be inserted into the carotid artery caudal to the transplant. Hence there would seem to be reasonable grounds for optimism that study of this preparation will permit resolution of some of the complexities arising from the results of the experiments reported in this paper.

SUMMARY

1. The parotid salivary $Na^+:K^+$ ratio of sheep with permanent unilateral parotid fistulae fell in the normal manner in response to Na^+ depletion, even though concurrent deprivation of water caused the plasma Na^+ concentration to rise above the concentration in the control period. This finding was made

whether the onset of water depletion preceded Na^+ depletion, was concurrent with it or occurred after a substantial Na^+ deficit was established.

2. Na^+-depletion experiments, in which sheep were fed on an artificial diet with low K^+ content, succeeded in dissociating the parotid response from the direction of change of the plasma K^+ concentration and the plasma $Na^+:K^+$ ratio. There was evidence that the parotid response was largely independent of change in the whole blood pH, the plasma HCO_3^- or the plasma Cl^- concentrations.

3. During both the water-depletion and the low-K^+-diet experiments, ipsilateral intracarotid ACh infusions were made at various stages of Na^+ deficit. This raised the parotid secretion rate to the control period level or higher, yet the salivary $Na^+:K^+$ ratio was greatly reduced below that found during normal Na^+ balance. Hence this fall of the salivary ratio was attributed to an increased secretion of adrenal hormones, and not to a decrease of salivary secretion rate.

4. Comparison of water-depletion and water-loading experiments indicated that the parotid response to Na^+ depletion was not determined by the concentration of antidiuretic hormone in the blood.

5. Na^+ depletion in a thyroidectomized sheep caused the salivary $Na^+:K^+$ ratio to fall in the normal manner.

6. A reason is advanced for proposing that the finding of a normal parotid response to Na^+ depletion, even though the plasma Na^+ concentration rises because of concurrent water deficit, does not necessarily exclude the usual fall of plasma Na^+ concentration as the specific stimulus to the putative receptor controlling output of electrolyte-active adrenal steroids.

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THE ALANINE AND OXO ACID CONCENTRATIONS IN MESENTERIC BLOOD DURING THE ABSORPTION OF L-GLUTAMIC ACID BY THE SMALL INTESTINE OF THE DOG, CAT AND RABBIT *IN VIVO*

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It has been shown by Neame & Wiseman (1957) that the absorption of L-glutamic acid and aspartic acid by the dog's small intestine *in vivo* results in an increased alanine concentration in the blood passing through the intestine, and their findings suggest that when protein is being digested and glutamic acid liberated in relatively low concentration in the intestinal lumen almost all the glutamic acid is involved in the transamination reaction. These observations explain the finding of a lower level of glutamic acid than was expected in the portal blood after feeding casein to dogs (Dent & Schilling, 1949) and an examination of their chromatogram shows an unexpected increase in blood alanine.

The study of transamination by the small intestine *in vivo* has now been carried further and the experiments described below show that the phenomenon also occurs in the cat and rabbit. The mesenteric blood concentrations of pyruvate and α-oxoglutarate have also been investigated during the absorption of glutamic acid by the dog, cat and rabbit, but no significant relationship was found between any changes in their concentrations and the transamination that was occurring.

METHODS

Experimental procedure

Dogs weighing between 8 and 22 kg and cats and rabbits weighing between 1·6 and 3·0 kg were used, and were allowed only water during the 24 hr before the experiment. Pentobarbitone sodium B.P. was used as the anaesthetic, the dogs receiving about 50 mg/kg body weight (intravenously), the cats about 50 mg/kg body weight (intraperitoneally), and the rabbits about 60 mg/kg body weight (intravenously). Artificial respiration via an intratracheal cannula was maintained throughout all experiments by means of an 'Ideal' pump (C. F. Palmer, Ltd., London) and amounted to about 0·5 l./kg body weight/min in the case of dogs, about 0·6 l./kg body weight/min in the case of cats, and about 0·7 l./kg body weight/min for rabbits. The artificial respiration was begun as soon as possible after anaesthesia had been induced.

The abdomen was opened by a mid-line incision, and in readiness for the taking of blood from the mesenteric venous trunk the latter was located and carefully cleared distal to its junction with the gastrosplenic vein. The mesenteric artery was then cleared and a loose thread ligature placed around it ready for ligation towards the end of the experiment (see below). The abdominal aorta was next cleared ready for the taking of an arterial blood sample. Then the small intestine was ligated in all three animals at about the middle of the duodenum, this being the approximate dividing line between blood draining into the gastrosplenic vein and blood draining into the superior mesenteric vein. The small intestine was further ligated just proximal to the ileocaecal junction in the case of the dog and cat, and in the case of the rabbit at a point level with the tip of the appendix, which lies in conjunction with the lower end of the ileum in this animal. A cannula was introduced into each end of the experimental portion of the small intestine (the part between the ligatures) and isotonic saline (at 37° C) was used to wash out the intestinal lumen. Any residual saline was then gently expelled from the intestinal lumen, a ligature was tied around each end of the experimental portion of intestine so as to exclude the cannulae, and the cannulae were then removed.

In the dog and cat the mesenteric venous blood collected comes from the small and large intestine, though mainly from the small intestine. In the rabbit, however, a large part of this mixed blood is derived from the large intestine, which, in this animal, has a relatively large bulk, and therefore the blood from the experimental portion of intestine may be considerably diluted by blood from the large intestine. In view of this, two series of experiments were carried out on the rabbit. In one series both the large and small intestine were left *in situ*, while in the other series that part of the intestine distal to the experimental portion of small intestine was removed immediately after opening the abdomen. In practice it was found necessary to leave *in situ* a short length of the colon, some of which has a mesentery in common with part of the small intestine.

In all except control experiments a neutralized 2% L-glutamic acid solution (6–10 ml./kg body weight) at 37° C was introduced into the upper end of the lumen of the experimental portion of intestine and gently massaged down the intestine so as to distribute it evenly. The abdominal wall was then closed after a thermometer had been inserted, and the animal left undisturbed for 30 min. At the end of this period the abdomen was reopened, the closed tip of a pair of Doyen's scissors passed under the portion of mesenteric vein previously cleared, and the vein raised on the blunt edge of the scissors so as to obstruct any back-flow of blood along the mesenteric vein during collection of the venous sample. The blood was drawn from the mesenteric vein into a syringe moistened with heparin solution. When sufficient had been obtained (10–20 ml.) the vein was clamped and the ligature round the mesenteric artery tied to prevent loss of blood into the intestinal vessels (although this was not necessary in the case of the dog). About 1 ml. of the blood taken (to be used for oxo acid estimation) was transferred rapidly into each of three 15 ml. weighed centrifuge tubes, each containing 3 ml. of distilled water and 4 ml. of 10% trichloroacetic acid. The exact volume of blood added to each tube was determined by weighing. The remainder of the venous sample (to be used for glutamic acid and alanine estimations) was not deproteinized until after an arterial blood sample had been taken from the abdominal aorta, and after the experimental portion of intestine had been removed and drained of its contents. The time interval between the taking of the mesenteric venous sample and the arterial sample was usually 1·5–2 min and never more than 2·5 min. In view of the instability of pyruvate in freshly drawn blood (Bueding & Wortis, 1940; Long, 1944) the exact time interval between the venepuncture and the deproteinization of the venous blood was noted; the time between the taking and the deproteinization of the arterial blood was made to correspond with this interval in order to compensate for any losses in the pyruvate between the drawing of the blood and its deproteinization. This time interval was usually between 1 and 1·5 min and never more than 2 min.

In control experiments the same procedure was carried out, except that the small intestine was left empty after the initial washing out with saline.

Chemical methods

Amino acid solutions. The L-glutamic acid was a commercial sample (L. Light and Co., Ltd.) used without further purification, and gave the theoretical yield of gas on decarboxylation. It was dissolved in distilled water with the aid of NaOH and the final solution was adjusted to about pH 6 (chlorophenol red as indicator).

Treatment of blood samples. The initial treatment of the blood samples for the estimation of oxo acids has been described above. After weighing the tubes to which the blood had been added, the deproteinized blood in each tube was thoroughly mixed, centrifuged at 2500 r.p.m. for 10 min and the supernatant filtered. Blood for glutamic acid and alanine estimation was well stirred and deproteinized as described by Neame & Wiseman (1957).

Recovery of residual amino acids from the intestinal lumen. At the end of each experiment the experimental portion of intestine was drained and washed out with isotonic saline. A sample was deproteinized and its glutamic acid content estimated as described by Neame & Wiseman (1957).

Estimation of amino acids. L-Glutamic acid was estimated by the manometric method of Gale (1945) and alanine by the colorimetric method of Alexander & Seligman (1945) as modified by Christensen, Riggs & Ray (1952).

Estimation of oxo acids. Pyruvic acid and α-oxoglutaric acid were estimated by the method of Goodwin & Williams (1952) with certain modifications. 6 ml. of each blood filtrate was pipetted into a 50 ml. separating funnel, which was then brought to 25° C in a water-bath (10 min being needed for this purpose). 1 ml. of 0·1% (w/v) 2:4-dinitrophenylhydrazine in 2 N-HCl was added and the separating funnel allowed to stand at room temperature for 25 min. The 2:4-dinitrophenylhydrazones of the oxo acids were then extracted with 4 ml. of diethyl ether by shaking for 1 min. The two layers were allowed to separate and the aqueous (lower) layer discarded. In some cases the ethereal layer was not clear but, after draining off most of the aqueous layer, clearing could be induced by giving two or three firm shakes to the separating funnel. The ethereal layer was extracted with 4 ml. of 15% (w/v) Na_2CO_3 by shaking for 1 min and the Na_2CO_3 layer transferred to a test-tube by means of a Pasteur pipette. 2 ml. of this was treated with 1 ml. of 3 N-NaOH and the optical density was estimated in a Hilger Uvispek spectrophotometer at 414 mμ (isosbestic point) and at 443 mμ (maximal absorption of pyruvic acid 2:4-dinitrophenylhydrazone). Standard solutions of pyruvate and α-oxoglutarate were taken through this entire procedure and all oxo acid estimations carried out on the same day as the experiment. The values for total oxo acid concentrations obtained from replicate samples were usually within 1%.

Paper chromatography of oxo acids. The remainder of each Na_2CO_3 extract was acidified by the dropwise addition of conc. HCl until effervescence no longer occurred and the yellow colour of the solution had become pale. The 2:4-dinitrophenylhydrazones were then extracted with 4 ml. of diethyl ether and the ethereal layer transferred to a shallow tube, evaporated to dryness *in vacuo* at room temperature, and stored at − 20° C until required for chromatography. (Evaporation to dryness is effected more rapidly if the last stages of the acidification are carried out carefully with dilute HCl.) The 2:4-dinitrophenylhydrazones were dissolved in about 0·5 ml. of diethyl ether, and 0·1 ml. applied to Whatman No. 1 filter paper and dried in a stream of warm air. Ascending chromatograms were run for 36 hr using butanol saturated with N-NH_4OH as solvent (Cavallini, Frontali & Toschi, 1949).

Oxo acid standards. Commercial samples (L. Light and Co. Ltd.) of sodium pyruvate and α-oxoglutaric acid were used as standards without further purification. The purity of the sodium pyruvate was found to be 81% by the manometric method described by Umbreit, Burris & Stauffer (1949). The purity of the α-oxoglutaric acid was assessed by the manometric method of Krebs (1950), and found to be 97%.

Identification of parts drained by the vein used for venepuncture

The animals used for this purpose were killed by an overdose of pentobarbitone sodium B.P. administered as described previously. The abdomen was opened, the mesenteric vein identified and a loose ligature tied around it. The vascular system of the intestine was washed out with isotonic saline introduced into the aorta or mesenteric artery (using a pressure of about 140 mm Hg), depending upon the size of the animal. A cannula connected to a pressure bottle containing blue Neoprene Latex (B. B. Chemical Co., Ltd., Leicester) from which all air bubbles were carefully excluded, was then inserted into the portal vein as near the liver as possible. A pressure of about 20 mm Hg was then exerted on the Neoprene Latex to induce passage into the intestinal vessels. As soon as the Neoprene Latex had reached the antimesenteric side of the intestine the pressure was released, a ligature tied around the portal vein distal to the tip of the cannula, and the cannula removed. The stomach, intestine and spleen were then removed *in toto* and placed in a 5% solution of formaldehyde acidified with HCl (the pH being about 2). After 2–4 hr the specimen can be dissected and the vessels exposed and examined. The site used for venepuncture for the blood samples during an absorption experiment is easily identified.

RESULTS

The results show that during the absorption of glutamic acid by the small intestine of the dog, cat and rabbit there is an increase in the concentration of alanine in the mesenteric blood as it passes through the small intestine. The phenomenon can be easily demonstrated in the dog and cat even when the large intestine is left *in situ* (as is shown by the results in Tables 1 and 2). In the case of the rabbit, however, when the entire intestine is left *in situ* the difference between the alanine concentration in the arterial and the mesenteric venous blood during the absorption of glutamic acid shows either no change (Table 3; Expts. 1–3 and 6) or a definite rise (Table 3; Expts. 4, 5 and 7). However, when the large intestine of the rabbit is removed before the experimental period has begun the mesenteric venous blood shows a much greater

TABLE 1. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the dog. (Small and large intestine *in situ*. In the control experiments the small intestine was left empty.)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/ kg body wt.)	% of amount introduced
1	133	151	43	41	0	0	4649	580	87
2	56	79	22	22	4	5	11430	820	65
3	79	113	23	23	2	1	9250	840	69
Control 1	75	66	41	37	1	1	—	—	—
Control 2	42	46	9	9	0	0	—	—	—

TABLE 2. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the cat. (Small and large intestine *in situ*. In the control experiments the small intestine was left empty.)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/ kg body wt.)	% of amount introduced
1	78	124	19	26	5	5	1403	830	94
2	108	169	21	24	2	2	2320	800	86
3	25	51	10	11	1	1	1706	1070	95
Control 1	47	40	14	12	1	1	—	—	—
Control 2	64	63	15	16	1	2	—	—	—

alanine concentration than the arterial blood during the absorption of glutamic acid (Table 4). The reason for this variability in the experimental findings in the case of the rabbit when the entire intestine is left *in situ* is that the contribution of venous drainage from the large intestine to the common mesenteric vein is so much greater in the rabbit than in the dog or cat. Determination of the parts of the intestine drained by the vein from which venous blood samples were collected showed that in the dog and cat the vein used for venepuncture drained mainly the small intestine, and only a small amount of blood came from the large intestine. In the rabbit, however, where the large intestine comprises much of the bulk of the total intestine, the venous drainage from the small intestine appeared to be only slightly greater than the venous drainage from the large intestine. In the experiments carried out on the rabbit after the removal of the large intestine, blood from the small intestine was no longer diluted by blood from the large intestine. A rise in alanine concentration in the blood passing through the intestine could then be clearly demonstrated. The results with the rabbit show the importance of determining the area drained by the common mesenteric vein in this type of experiment.

Table 3. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the rabbit. (Small and large intestine *in situ*. In the control experiment the small intestine was left empty)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/ kg body wt.)	% of amount introduced
1	126	124	66	34	3	5	1090	450	41
2	119	119	53	46	2	3	998	590	38
3	92	99	37	33	2	2	1450	630	52
4	130	147	50	40	7	6	1440	480	43
5	135	163	65	48	2	3	1600	670	52
6	154	159	62	49	3	5	1260	440	37
7	131	181	68	55	2	2	1690	840	62
Control	99	90	53	37	2	1	—	—	—

Table 4. Alanine, pyruvate and α-oxoglutarate concentrations in arterial (Art.) and mesenteric venous (Mes.) blood taken 30 min after the introduction of 2% L-glutamic acid solution into the lumen of the small intestine of the rabbit. (Only small intestine *in situ*. In the control experiments the small intestine was left empty)

	Alanine (μmole/100 ml.)		Pyruvate (μmole/100 ml.)		α-Oxoglutarate (μmole/100 ml.)		Glutamic acid absorbed		
Expt. no.	Art.	Mes.	Art.	Mes.	Art.	Mes.	(μmole)	(μmole/ kg body wt.)	% of amount introduced
1	104	153	36	30	10	11	935	580	42
2	217	286	87	57	9	6	2350	940	67
3	208	297	45	25	9	10	1910	710	60
4	153	230	67	41	7	13	1460	660	50
Control 1	127	124	76	53	4	3	—	—	—
Control 2	125	114	40	32	4	4	—	—	—

In control experiments with the small intestine empty there was no appreciable difference in the alanine concentration between the arterial blood and mesenteric venous blood.

In some of the experiments on dogs, cats and rabbits in which the whole intestine was left *in situ*, glutamic acid was determined in both arterial and venous blood at the end of the experimental period. This was not possible in experiments on rabbits with the large intestine removed, owing to insufficient blood. In the dog the mesenteric venous concentration of glutamic acid was from 25 to 83 μmole/100 ml. more than that in the arterial blood. With the cat the mesenteric venous concentration was from 57 to 144 μmole/100 ml. higher than the arterial level. In both these animals the arterial concentration of glutamic acid at the end of the experiments was about 140 μmole/100 ml., compared with a value of about 90 μmole/100 ml. in the control experiments. In the rabbit with the whole intestine *in situ* there was no significant difference between the arterial and mesenteric venous concentrations of glutamic acid at the end of the experimental period. These results show that, with the amounts of glutamic acid introduced into the lumen of the intestine in these experiments, some glutamic acid does appear in the blood passing through the intestine of the dog and cat.

As the glutamic acid left in the experimental portion of intestine at the end of the experimental period was determined, the amount which had disappeared from the lumen during this period could be calculated. In the case of the dog it was found that an average of 74% of the glutamic acid introduced had disappeared by the end of 30 min; in the cat an average of 92% disappeared, and in the rabbit an average of 49% disappeared.

The concentrations of pyruvate and α-oxoglutarate in the mesenteric venous blood from the experimental portion of intestine were not appreciably different from their concentrations in the arterial blood, except in the case of the rabbit, where the arterial pyruvate concentration was higher than the mesenteric venous concentration under both experimental and control conditions.

The oxo acid 2:4-dinitrophenylhydrazones derived from the blood samples collected at the end of the experimental periods were examined by one-dimensional paper chromatography. It was found that only pyruvate and α-oxoglutarate were present in arterial and venous blood collected both from control loops of intestine and from intestine absorbing glutamic acid.

DISCUSSION

The results show that when glutamic acid is absorbed from the small intestine of the dog, cat and rabbit, a rise in the concentration of alanine occurs in the blood passing through the intestine. This is clearly demonstrable in the case of the cat and the dog in spite of dilution of the blood from the small intestine with blood from the large intestine. In the rabbit, however, the difference between the concentration of alanine in the mesenteric venous blood and in the arterial blood varies considerably from one experiment to another when the whole intestine is left *in situ*. This is probably due to a variable dilution of the blood from the small intestine by the comparatively large volume of blood flowing from the large intestine. During glutamic acid absorption in the rabbit with the large intestine removed the mesenteric venous concentration of alanine was always considerably higher than the arterial concentration and the rise in alanine content of the blood was greater than in the experiments with the dog and cat.

The concentrations of pyruvate and α-oxoglutarate in the blood passing through the intestine are unchanged by the transamination that occurs after introducing glutamic acid into the intestinal lumen. A consideration of the transamination reaction suggests that the rise in alanine concentration in the venous blood would be accompanied by an increase in α-oxoglutarate concentration and a concomitant fall in the pyruvate concentration, but this was not supported by the experimental results. A fall in venous pyruvate concentration occurred in the rabbit only and was unrelated to the rise in alanine concentration, since it occurred even in control experiments when no glutamic acid was introduced into the intestinal lumen.

From these results it would appear that the normal metabolism of the intestinal cells overshadows or compensates for any changes in the oxo acid concentrations of the blood brought about as a result of the transamination of the absorbed glutamic acid. The alanine added to the blood during its passage through the glutamic acid-absorbing intestine is usually considerably more than the amount of pyruvate found in the arterial blood supplying the intestine and must come from the general metabolism of the intestinal cells. If all the α-oxoglutarate produced as a result of the transamination appeared in the mesenteric venous blood its concentration would be increased about tenfold. The site of the transamination in the intestine is as yet undetermined, but the most likely position would be in the mucosal epithelial lining.

It is stimulating to find that a phenomenon first discovered from *in vitro* experiments on the rat intestine (Matthews & Wiseman, 1953) is now shown to occur *in vivo* in the dog (Neame & Wiseman, 1957), cat and rabbit.

SUMMARY

1. The absorption of L-glutamic acid by the small intestine of the dog, cat and rabbit has been studied *in vivo*.

2. The absorption of glutamic acid is accompanied by a rise in the concentration of alanine in the blood passing through the intestine in all three animals. In the rabbit this can only be demonstrated consistently after removal of the large intestine.

3. The concentrations of pyruvate and α-oxoglutarate in the blood passing through the intestine in the dog and cat remain unaffected by the absorption of glutamic acid and the transamination involved.

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A METHOD FOR STUDYING THE EFFECTS OF IONS AND DRUGS ON THE RESTING AND ACTION POTENTIALS IN SMOOTH MUSCLE WITH EXTERNAL ELECTRODES

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A number of papers have dealt with the effects of drugs on the rate and height of action potentials in smooth muscle (cf. Bozler, 1948; Bülbring, 1956), but there are relatively few descriptions of changes in the resting potential. This is probably because an external electrode technique has not been available and resting potential measurements with internal electrodes are difficult to make, owing to the small size and spontaneous motility of the smooth muscle fibres (Bülbring, 1955).

A few years ago Stämpfli (1954) described a relatively simple method for measuring the full value of the resting potential with external electrodes. His method is based on the theoretical calculation that the full value of the membrane potential can be measured with external electrodes on a core conductor, when the short-circuiting is negligible (Hodgkin & Rushton, 1946). Stämpfli obtained this condition by increasing the outside resistance of the preparation in the interpolar region by replacing most of the ions in the interstitial fluid with a nearly ion-free sucrose solution. This method has so far been applied to bundles of myelinated nerve fibres (Stämpfli & Straub, 1954), and to non-medullated fibres (Ritchie & Straub, 1956, 1957), where action potentials were also recorded. The present paper deals with the application of this technique to smooth muscle.

METHODS

Most experiments were done on smooth muscle strips taken from the taenia coli of the guinea-pig. They were 10–15 mm long and 0·5 mm in diameter. The strips contracted strongly on removal from the gut but were stretched to their '*in situ*' length when mounted in the apparatus. At this size the taenia coli exhibits little tension (Bülbring, 1955). In addition, a number of preparations were obtained from the longitudinal muscle coat of the small intestine of the pike, frog, rabbit, rat, guinea-pig and cat and of the rectum of the trout. These preparations were dissected according to the method described by Evans & Schild (1953). All strips used were teased off from larger pieces and never cut to size. Not more than 10 min were required to dissect and mount the preparations in the apparatus. The results obtained with preparations from different animals were so similar that they have not been dealt with separately.

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The strips were mounted in the apparatus described by Stämpfli (1954), and in more detail by Straub (1956*a*) so that they lay in a horizontal insulating tube containing isotonic sucrose solution, while each end was suspended in a vertical tube through which Locke's solution flowed. A pair of Ag–AgCl electrodes, connected over a KCl–Agar bridge to the Locke's solution, recorded the potential difference between the two sucrose–Locke junctions. The electrodes were connected to a pair of cathode followers and the potential difference, amplified by a direct-coupled amplifier, was displayed on a cathode-ray tube, and recorded on a moving film.

The Locke's solution used for the mammalian preparations contained (mM) NaCl, 154; KCl, 5·6; $CaCl_2$, 2·2; $NaHCO_3$, 1·9; dextrose, 5. The Ringer's solution used for preparations taken from cold-blooded animals contained (mM) NaCl, 119; KCl, 1·9; $CaCl_2$, 1·1; $NaHCO_3$, 2·2; dextrose, 5. Solutions with higher potassium concentration were obtained by replacing an equivalent amount of sodium chloride by potassium chloride. In addition, a 'sulphate-Locke's solution' was used, in which the chloride ions had been replaced by sulphate ions and the calcium increased to 10 mM. It was necessary to add an excess amount of calcium to the sulphate-Locke's solution so that in the final solutions, made up by mixing Locke's with sulphate-Locke's solution, the amount of free calcium ions calculated from the solubility product $6{\cdot}1 \times 10^{-5}$ (Landolt & Börnstein, 1923) varied by not more than 0·5 mM, except in solutions with less than 80 mM chloride. Throughout the whole course of the experiment all solutions were equilibrated with 95% O_2 and 5% CO_2. The pH of the Locke's and the sulphate-Locke's solution was 6·5, that of the Ringer's solution 7·0. The isotonic sucrose solutions flowing through the horizontal insulating tube of the apparatus contained sucrose (A.R.), 10% (w/v) for mammalian preparations and 7·3% for cold blooded preparations, in distilled water which had been passed over ion-exchange resin. Both had a specific resistance of more than 10^6 Ωcm. The fluid in the vertical tube on one side of the chamber was kept at room temperature (17–20° C.) so that the muscle remained inactive, thus maintaining a stable potential against which the test solution in the other vertical tube, kept at body temperature, could be compared.

RESULTS

Assuming that the smooth-muscle preparation acts as a core conductor when mounted between two recording electrodes, an increase of the outside resistance produced by the flow of the sucrose solution would be reflected by the increase in the amount of depolarization produced by a standard potassium chloride solution applied to one side of the preparation. This supposition was tested in four experiments where it was found that the depolarization increased during the first 25 min, and during the next 4 hr it declined slowly to about two-thirds of its peak value. This implied that the penetration of the sucrose solution was near completion after the first 25 min, while the decline which followed was probably due to a progressive deterioration of the preparation. For this reason no records were taken from muscle strips exposed for more than 4 hr.

When the preparations were placed in the apparatus at 37° C they usually showed bursts of uneven spike activity as shown in Fig. 1. In some experiments, however, the spikes became larger and more regular. The shape of the spikes was similar to that described by Bülbring (1957) and Holman (1957*a*) showing an initial slow phase of depolarization and a rapid repolarization with after-hyperpolarization.

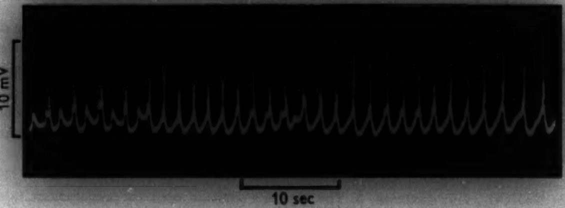

Fig. 1. Spontaneous irregular spike activity recorded from a strip of guinea-pig taenia coli by the sucrose-gap method. Temperature 35° C.

Effects of changes in ion concentration

Potassium ions. Solutions with a high potassium concentration produced a depolarization which was almost complete within 10–20 sec. On returning to Locke's solution an initial rapid repolarization was followed by a slow rise in potential which lasted about 60 sec until the original level was reached. A characteristic trace of such an experiment is shown in Fig. 2.

Preparations were bathed in a series of solutions containing different potassium concentrations. The relation between the depolarization produced and the logarithm of the potassium concentration is shown in Fig. 3, where the values obtained from a rabbit taenia coli and a trout rectum are plotted. The measurements were taken after the preparation had been bathed in the new solution for about 3 min. Table 1 summarizes results from eight experiments on various preparations showing depolarization by isotonic potassium chloride. The depolarization produced by isotonic potassium chloride was not more than 30 mV, and the steepest part of the curve in Fig. 3 gives a slope of 26 mV per tenfold change in potassium concentration.

When, instead of isotonic potassium chloride, potassium sulphate was used, a much greater depolarization was obtained (Table 1). The value of 51·6–60·6 mV which we found is close to the resting potential of 60 mV, measured with internal electrodes for *in situ* length taenia coli (Bülbring, 1954).

Apart from the changes in resting potential produced by increase in potassium concentration, fast regular spike activity developed gradually, decreasing in amplitude as the depolarization proceeded. After a potential fall of about 12 mV no further spike activity was observed and on recovery in Locke's solution the spikes reappeared as illustrated in Fig. 4.

Chloride ions. When the effect of decreasing the chloride concentration was investigated, chloride was substituted by sulphate, since it is known for

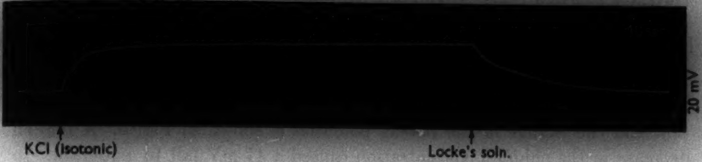

Fig. 2. Influence of isotonic potassium chloride on the resting potential recorded from a longitudinal muscle strip from the pike intestine. Upward deflexion represents depolarization. Temp. 18° C.

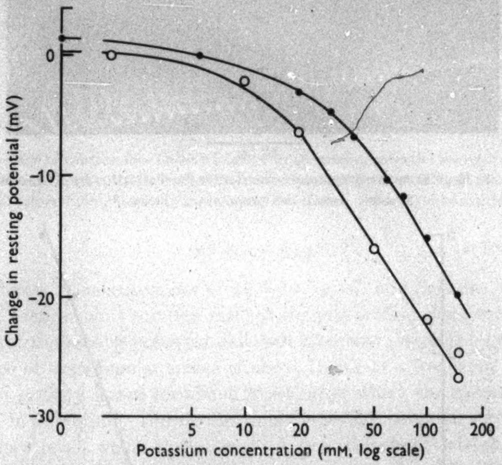

Fig. 3. The relationship between the logarithm of the potassium concentration and the depolarization produced in (*a*) rabbit taenia coli (●), and (*b*) longitudinal muscle strips from the trout rectum (○). Slope per tenfold change = 26 mV. Temp. 18° C.

TABLE 1. Depolarization produced by isotonic potassium chloride and sulphate.

Preparation	Temp. (°C)	Depolarization (mV) in isotonic KCl solution	Depolarization (mV) in isotonic K_2SO_4 solution
Trout rectum	16	23·6 (17·2–32·5)	—
Trout rectum	18	26·2	—
Pike intestine	16	19·3 (13·1–23·6)	—
Rabbit taenia coli	22	23·9 (20·0–30·5)	55·2 (51·7–58·7)
Guinea-pig taenia coli	25	21·3 (16·5–24·8)	56·1 (51·6–60·6)
Guinea-pig taenia coli	35	22·0	54·0
Guinea-pig taenia coli	35	20·0	—

striated muscle that this larger anion does not diffuse through the membrane (Boyle & Conway, 1941; Hodgkin & Horowicz, 1957). The solutions we used were made up by mixing different proportions of Locke's solution with one in which all the chloride ions were replaced by sulphate ions.

Solutions with a decreased chloride concentration caused a rapid depolarization which was complete in 1 min. The relation between the chloride concentration and the change in the resting potential is shown in Fig. 5. A tenfold decrease in the chloride concentration caused a 41 mV fall in potential. In addition to the change in resting potential, chloride concentrations of 100 and 75 mM produced bursts of regular spike activity.

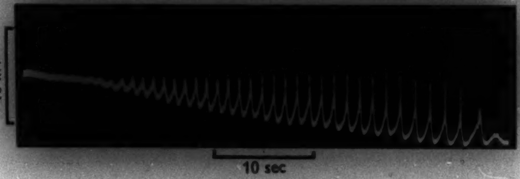

Fig. 4. Regular recovery spikes produced after depolarization by KCl solution (80 mM). Taenia coli preparation. Temp. 35° C.

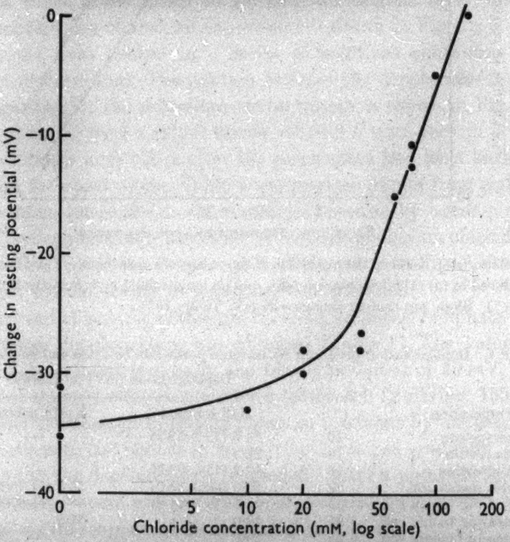

Fig. 5. The relationship between the logarithm of the chloride concentration and the depolarization produced in guinea-pig taenia coli. Slope per tenfold change = 41 mV. Temp. 18° C.

Lack of calcium ions. The effect of calcium-free Locke's solution was a depolarization amounting to about 13 mV. A typical experiment is illustrated in Fig. 6. This figure shows that the time for complete depolarization was about 1 min, whereas the recovery in Locke's solution took only about 30 sec. The resting potential recovered to a value 5 mV higher than the initial level in Locke's solution. The changes in polarization seen in Fig. 6 are similar to those observed for nerve fibres by Stämpfli & Nishie (1956) and by Straub (1956*b*).

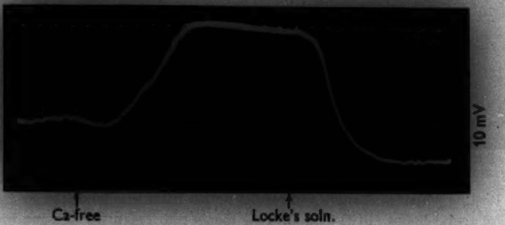

Fig. 6. The effect of calcium-free Locke's solution on the resting potential recorded from guinea-pig taenia coli. Temp. 18° C. The inserted base line signifies the position of the mean resting potential before the application of the calcium-free solution; deflexion upwards, depolarization.

Effects of drugs

Acetylcholine. Concentrations of 10^{-6}–10^{-7} g/ml. acetylcholine were used. When the acetylcholine solution reached the preparation there was an initial slight fall of the resting potential, followed after some seconds by the sudden appearance of large regular spikes of about 12 mV at a frequency of 1·6/sec. This spike activity lasted for about 20 sec, after which the spikes gradually decreased in amplitude. During this time the resting potential showed a slow fall to a new level, where it remained. A typical record is shown in Fig. 7, taken from an experiment in which there was no spike activity before the application of the acetylcholine.

When some spike activity was already present before the application of the drug, the main effect of actylcholine consisted of an increase of spike frequency from 0·9 to 1·6/sec, together with depolarization (Fig. 8). This is the effect which has been described by Bülbring (1957) with intracellular electrodes.

In five experiments a depolarization ranging from 4 to 8 mV was produced by acetylcholine (10^{-6}). When the preparation was then washed in Locke's solution, the potential slowly returned to its original level or to a somewhat higher value.

Choline. In one experiment (Fig. 9) a solution was used in which all the sodium chloride was replaced by an equivalent amount of choline chloride. This solution produced an initial depolarization of 10 mV, followed after 1 min by a burst of regular spikes lasting 40 sec. During this burst of activity the resting potential was still low but afterwards it increased gradually. It is rather surprising that spikes occur in a sodium-free solution. This finding suggests that smooth-muscle fibres may produce action potentials in a similar way to that described by Fatt & Katz (1953) for crab muscle, where large spikes were observed in sodium-free 'choline-Ringer'. An obvious question,

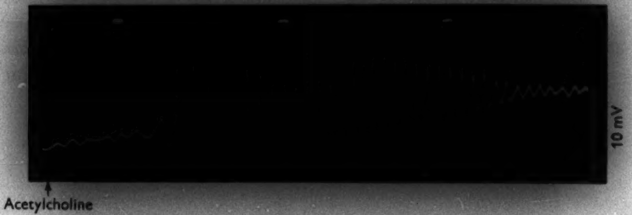

Fig. 7. Resting potential changes and regular action potentials produced by the application of acetylcholine (10^{-6}) on guinea-pig taenia coli. Temp. 34° C; time 10 sec.

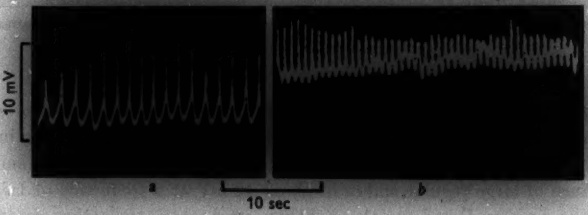

Fig. 8. (*a*) Effect of ACh (10^{-7}) on guinea-pig taenia coli, showing spontaneous activity. (*b*) Result after 3 min where there was a depolarization of 6·5 mV and a marked increase in spike rate. Temp. 36° C.

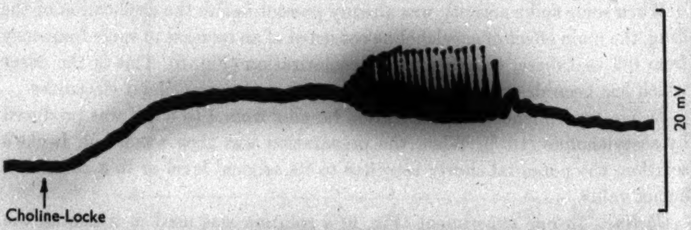

Fig. 9. Change in resting potential and regular spikes produced by the application of a sodium-free 'choline-Locke's' solution to guinea-pig taenia coli. Temp. 35° C, time 10 sec.

as already pointed out by Fatt & Katz, is whether there may not have been retention of sodium ions in the space immediately surrounding the fibre membrane. This question cannot yet be definitely answered. In the same experiment, however, the effect of other solutions was complete in 30 sec. Similar results with low sodium were recently obtained on smooth muscle by Holman (1957 *a*). She found that the spikes disappeared only after a prolonged soaking in sodium-free solutions, and that normal spikes still occurred in a solution containing only 17 mM-Na.

DISCUSSION

Our results show that the method for measuring resting potentials described by Stämpfli (1954) is applicable to smooth muscle. It was possible to measure the changes in the resting potential produced by ions and drugs. The values found were similar to those measured by the micro-electrode technique (Bülbring, 1954, 1956; Holman, 1957 *a*). In addition, we recorded spike activity, the frequency and height of which was altered by drugs and ions. Our technique allows a quick change of the bathing solution, since the solutions are continuously flowing. Therefore the whole sequence of changes in resting potential and spike activity caused by the test solutions could be continuously recorded.

The question naturally arises, how can one record from smooth muscle with external electrodes 7 mm apart, when the muscle between the electrodes is bathed in a high-resistance sucrose solution. As the length of a single muscle fibre is only from about 50 to 100 μ (E. Bülbring, personal communication) the recording electrodes are separated by a large number of muscle fibres bathed in the sucrose solution. One possibility is that the sucrose solution fails to replace all the ions lying in the inter-fibre spaces, so that a low-resistance bridge exists between them. Another possibility is that there is some kind of electrical connexion between the individual fibres, since it is known that smooth-muscle fibres do not behave as single independent units. For instance, Engelmann (1870) showed that there is a correlation between the contraction of different fibres. This is also seen by the propagation of action potentials along smooth muscle which, according to Bozler (1948), cannot be explained by a nervous mechanism. This hypothesis has recently been confirmed on nerve-free preparations by Evans & Schild (1953, 1956). Finally, Prosser, Sperelakis & Bergmann (1955) suggested that smooth-muscle fibres are linked by regions of low impedance. It may be on account of this property of smooth muscle that we were able to record potential changes with the sucrose-gap method.

One may argue that artifacts due to junction potentials complicate the interpretation of results obtained with this technique when measuring changes in resting potential produced by different ion concentrations. At the interface between the sucrose solution and the Locke's solution there is a junction potential of about 35 mV, as calculated from the formula given by Henderson (1907, 1908), and the ion-mobilities given in Landolt-Bönstein's tables (1936). Further, when isotonic potassium chloride is applied this junction potential drops to 3·5 mV, the potassium-treated side being negative. However, only a small fraction of the difference between these two junction potentials will be recorded if there is little short-circuiting by the external medium. The exact value of this fraction cannot be estimated as we lack detailed information about the value of the short-circuiting factor. For most of the effects we observed the junction-potential artifacts can almost certainly be excluded because: (1) concentrations of drugs, too small to alter the junction-potential, produced changes in the potential between the electrodes as high as 10 mV; (2) replacement of the Locke's solution by a calcium-free Locke's solution was followed by a 13 mV change of potential, although this does not appreciably alter the value of the junction potential; (3) replacement by 'choline-Locke's solution' resulted in a lowering of the recorded potential, whereas the junction potential is increased; (4) although replacement by sulphate solutions and solutions with increased potassium concentrations caused depolarization and the calculated change in the junction potential would be in the same direction, the depolarization in potassium chloride could not be to any great extent due to the junction potential, since the depolarization was found to show the steepest increase in the high potassium concentrations, whereas the calculated junction potential changed only very slightly at these concentrations.

The depolarization which we found with isotonic potassium chloride was relatively low but of the same order as the depolarization obtained by previous workers in corresponding experiments on smooth muscle. We found that the depolarization was not more than 30 mV, both for cold- and warm-blooded animals, and that the steepest part of the curve relating depolarization to the potassium concentration (Fig. 3) showed a 26 mV depolarization for a tenfold increase in the potassium concentration. Holman (1957*b*) observed a 32 mV fall per tenfold change in K concentration for taenia coli muscle using the micro-electrode technique. In frog striated muscle, however, Ling & Gerard (1950) found that isotonic potassium chloride produced an 80 mV depolarization and that a tenfold change of potassium resulted in a potential fall of 44 mV. Burgen & Terroux (1953) working on heart muscle recorded a 50 mV depolarization with isotonic potassium chloride and a 38 mV slope per tenfold change. If the resting potential were only governed by the distribution of potassium ions on either side of the membrane, a tenfold change in the outside potassium concentration should give a 58 mV potential change. The deviation of the measured membrane potential change from this theoretical value is known to be due to the permeability of the membrane to other ions than potassium, such as sodium or chloride (Hodgkin & Katz, 1949; Adrian, 1956). Recently Hodgkin & Horowicz (1957) found that the part played by chloride ions can become important when the potassium concentration is suddenly changed and the chloride left unaltered. In this condition the chloride may temporarily dominate the membrane potential, until the chloride concentration inside the fibre has changed in accordance with the potassium potential. This may explain why we found a relatively small fall in potential when only the potassium concentration was changed and the chloride remained at high constant level. The importance of chloride ions in maintaining the resting potential in smooth muscle explains also why a depolarization of the order of the resting potential could be recorded when the muscle was treated by isotonic potassium sulphate. We found under these conditions a depolarization of 54–58 mV, which is very close to the resting potential of 60 mV measured by Bülbring (1954) with intracellular electrodes.

Apart from the changes in the resting potential, we have observed both regular and irregular spikes. Although it is difficult to estimate the number of muscle cells from which action potentials are recorded, the irregular spike activity observed in Locke's solution suggests that records were being obtained from a number of cells firing off fairly independently. The finding that on the application of acetylcholine or potassium chloride the action potentials became large and regular suggests that the cells in contact with the electrodes were firing fairly synchronously. The largest spikes we recorded were about 14 mV, which is less than the height of the spikes recorded from a single fibre with intracellular electrodes (Holman, 1957*b*). This is not surprising, since we record from a large number of muscle fibres and even if they seem to fire synchronously, temporal dispersion of individual spikes and the occurrence of inactive fibres would reduce the height of the recorded spike. In spite of the fact that the recorded spikes are smaller than those obtained from single fibres the method we used is suitable for studying the effects of drugs and ions on the frequency and form of spike activity. It is clear that resting potential and spike activity are related, spikes disappearing as a depolarization develops beyond a certain critical level. Drug effects can be studied at different 'preset' resting potential values by a previous application of potassium, or the change in resting potential caused by a drug can be investigated by testing this drug in a solution with various amounts of ions. In this way more information may be obtained about the mode of action of drugs on smooth muscle.

SUMMARY

1. The application of the 'sucrose-gap' technique for recording resting and action potentials of strips of smooth muscle from the gut has been described.

2. Isotonic potassium chloride (150 mM) produced a depolarization of up to 30 mV. A 26 mV depolarization per tenfold change in the potassium concentration was observed. Isotonic potassium sulphate produced a depolarization of 55 mV.

3. Substituting sulphate for chloride decreased the resting potential by 35 mV and there was a depolarization of 41 mV per tenfold change in the chloride concentration.

4. Calcium-free Locke's solution produced a potential fall of 13 mV.

5. Irregular spike activity was observed in spontaneously active preparations, while regular spikes were obtained by application of potassium and acetylcholine.

6. Acetylcholine produced a depolarization of 4–8 mV.

7. Regular spike activity occurred also in sodium-free 'choline-Locke's solution'.

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EXPERIMENTS ON THE FATE OF HISTAMINE AND ACETYLCHOLINE AFTER THEIR INJECTION INTO THE CEREBRAL VENTRICLES

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Drugs injected into the lateral ventricles of the brain are known to produce profound and long-lasting effects, but it is not known how long they remain in the ventricular system and whether the long-lasting effects depend upon their persistence in the ventricular spaces.

In the experiments to be described an attempt was made to determine the fate of histamine and acetylcholine injected into the lateral cerebral ventricles of cats and dogs by assaying the amounts recovered at different times after the injection in washings of the cisterna magna and the ventricles, or in the epicerebral or spinal c.s.f. Further, the histamine absorbed into the blood stream was estimated by its effect in producing acid gastric secretion, and some observations were made on circulatory and respiratory effects produced by the intraventricular injections.

METHODS

Cats (2–3·5 kg) and dogs (5–17 kg) were anaesthetized with either pentobarbitone sodium (36 mg/kg) intraperitoneally or chloralose (8 ml. of a 1% solution/kg) intravenously.

Cannulation of the cat's ventricle. The head of the cat was securely supported by a head holder and raised about 10 cm above the plane of the operation table. The Collison cannula for intraventricular injection described by Feldberg & Sherwood (1953) was used, modified so that there was only one side hole at about 1 mm from its occluded tip. A track was created in the brain before insertion of the cannula, by the use of a 'pilot-guide' (Fig. 1*a*). It consists of a metal cylinder tunnelled centrally throughout, and carrying a screw thread on its lower narrower part, which serves to screw it into the skull, and a metal shaft, which is passed through the hole of the cylinder and through the brain until the tip reaches the ventricle.

Cannulation of the dog's ventricle. The method was the same, in principle, as in cats. However, on account of the greater variation in the size of the dog's brain the length of the cannula had to be varied through a wider range and therefore the cannula had to be frequently inserted and taken out before its tip rested in the ventricle. To prevent damage by this repeated procedure to the thread cut in the skull, a metal sleeve was screwed into the skull and the cannula screwed into the sleeve. The sleeve, which is illustrated in Fig. 1*b*, was introduced by A. C. Palmer (unpublished experiments) for cannulation of the cerebral ventricle of the sheep. The thread on the outer surface of the sleeve which serves to screw it into the skull was ¼ × 40 B.S.F., while the thread on its inner surface which takes the thread of the cannula was the usual 3 B.A. thread.

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Intraventricular injections. Histamine acid phosphate, acetylcholine chloride and eserine sulphate were slowly injected in a volume of 0·25 ml. of magnesium-free Tyrode solution, followed after about 20 sec by another slow injection of 0·25 ml. of Tyrode solution in order to wash in the drug. All values of histamine refer to the base and those of acetylcholine and eserine to the salt.

Collection of c.s.f. from the cisterna magna. The cannula used was a 3 cm long 20 S.W.G. hypodermic needle devoid of its butt and entirely surrounded by a closely fitting steel tube, except for the terminal 3 mm of the sharp end to be inserted into the cisterna. The other end was attached to a small length of polythene tubing and kept closed, during the times no fluid was collected, by a small glass stopper. In order to keep the cannula in position a pointed iron rod with a hole in its centre was first passed through the retracted muscles of the neck so that the hole faced the middle of the exposed atlanto-occipital membrane. The cannula was then passed through the hole of the iron rod and the membrane into the cisterna. On removal of the glass stopper, the cerebrospinal fluid started to flow out. In cats between 0·5 and 2·4 ml., and in dogs 2–3 ml. c.s.f. were collected before the flow stopped. To restart it and to wash out the ventricular spaces, Tyrode solution in amounts of 1 ml. in cats and 2 ml. in dogs was injected intraventricularly 5–6 times at about 2 min intervals. Each injection led to an outflow a little in excess of the injected volume, which was collected as a separate sample.

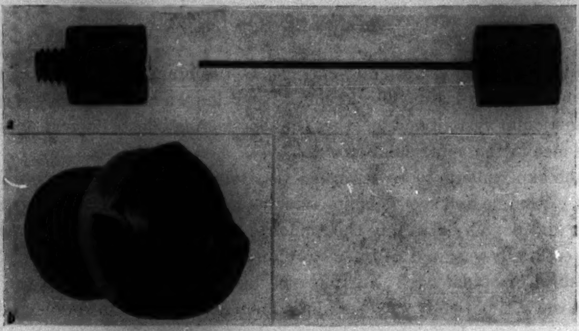

Fig. 1. Pilot guide (*a*) and sleeve (*b*) used for cannulation of the lateral cerebral ventricle.

In experiments using histamine the samples were assayed for histamine on the atropinized guinea-pig ileum; in those using acetylcholine, the assay was carried out on eserinized frog rectus muscle.

Collection of c.s.f. by lumbar puncture. Lumbar puncture was made by a 20 S.W.G. needle. About 0·3 ml. of c.s.f. could be collected in cats and about 2 ml. in dogs before the flow stopped. It could be restarted by intraventricular injections of 1–2 ml. of Tyrode solution. The butt of the needle could be closed by 'Plasticine' when samples were not being collected.

Collection of c.s.f. from epicerebral subarachnoid space. The ventricular cannula was on the right side and collection was effected through a hole bored in the skull at a corresponding point on the left side. On incision of the dura successive samples of 0·1–0·2 ml. were obtained.

Collection of samples of gastric secretion and estimation of their free HCl content. The procedure was that described by Edkins (1906). Cats were starved for 24 hr and anaesthetized by intraperitoneal pentobarbitone sodium. The abdomen was opened by a small median incision. The duodenum was tied off about 2 cm from the pylorus and a polythene tube was inserted into the stomach through a small cut on the antimesenteric border below the pyloro-duodenal junction. The stomach was washed out several times with 10 ml. warm saline solution introduced through the polythene tube and removed with a pipette, and then filled with 10 ml. of warm saline solution. The free end of the polythene tube was closed by means of a clamp and the solution allowed to remain in the stomach for 20 min. It was then removed and replaced with another 10 ml. of saline solution. This procedure was repeated every 20 min and the 20 min samples were titrated for their free HCl content against 0·01 N-NaOH using Töpfer's reagent as indicator. When the acid gastric secretion obtained on intraventricular injection was compared with that produced by slow intravenous infusion, the continuous slow injector (C. F. Palmer Ltd.) was used, and the histamine solution was infused through the femoral vein.

RESULTS

Circulatory and respiratory effects

In cats the intraventricular injection of 500 μg of either histamine or acetylcholine produced pressor and depressor effects. Typical instances are illustrated in Figs. 2 and 3. The effects occurred also after section of the vagi in the neck. Intraventricular injections of Tyrode solution were ineffective. When histamine produced a pressor effect it was always small and gradual as shown in Fig. 2A, but with acetylcholine the rise was often sharp and large, as shown in Fig. 3B. The depressor effect of histamine was also usually less pronounced and more transient than that of acetylcholine; it was sometimes followed by slow rhythmic variations in the arterial blood pressure. In addition, histamine produced tachycardia lasting for a few minutes, the heart rate increasing by up to 90 beats/min. When acetylcholine was injected together with 100 μg of eserine the depressor effect was more prolonged; it did not occur after intravenous atropine.

In dogs the intraventricular injection of 500 μg of histamine produced an initial transient gradual rise in arterial blood pressure often followed by slow rhythmic changes in blood pressure. There was no cardio-acceleration but respiration was sometimes stimulated. An intraventricular injection of 500 μg acetylcholine caused a small prolonged rise in arterial blood pressure and stimulation of respiration. A similar effect was obtained with eserine. If respiration was irregular, it became not only more frequent, but also regular. This is shown in Fig. 4.

The difference between the effects in cats and dogs is probably mainly due to the fact that in cats a small fraction of the injected histamine or acetylcholine is pushed into the subarachnoidal space, from where it would be quickly absorbed, whereas in dogs, with the much larger ventricular spaces, this would not occur. The finding that the depressor action in cats occurred usually after the second injection of 0·25 ml. Tyrode and that the depressor

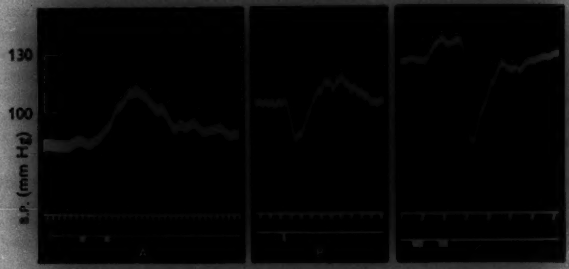

Fig. 2. Arterial blood pressure records from three cats. Effects of intraventricular injection of 500 μg of histamine. The two marks on the bottom line in A and C indicate the time of injection and of subsequent washing in of 0·25 ml. Tyrode solution; the single mark B indicates the end of washing in. In A and B, time marker 10 sec; in C, 30 sec.

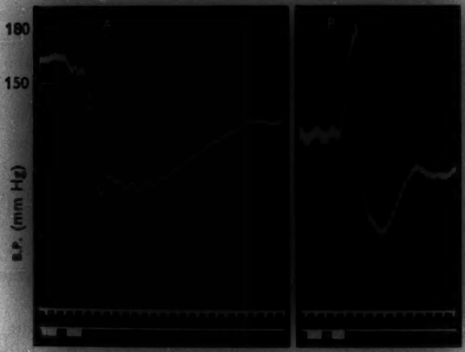

Fig. 3. Arterial blood pressure from two cats. Effects of intraventricular injections of 500 μg ACh. The two marks on the bottom line indicate the time of injection and of subsequent washing in of 0·25 ml. Tyrode solution. Time marker, 10 sec.

Fig. 4. Dog's arterial blood pressure recorded on a quick-moving drum to show individual heart beats and rate of respiration. Effect of intraventricular injection of 100 μg eserine. The second block 4 min after injection. The two marks on the bottom line indicate time of injection and of subsequent washing in with 0·25 ml. Tyrode solution. Time marker, 10 sec.

effect of acetylcholine did not occur after atropine would thus be easily accounted for. Since small doses of histamine injected intravenously increase the heart rate, the cardio-acceleration seen after intraventricular injection of histamine could also be an effect of absorbed histamine. On the other hand, the pressor effects and the stimulation of respiration are central in origin and so, probably, are the rhythmic changes in arterial blood pressure. A similar centrally produced blood-pressure effect has recently been described by Trendelenburg (1957) on injection of a small dose of histamine (10 μg) into the cerebral lateral ventricle of the anaesthetized cat.

Recovery in cats of injected histamine and acetylcholine from the cisterna magna

Cerebrospinal fluid collected from the cisterna had no effect on the atropinized guinea-pig's ileum but caused small contractions on the non-atropinized preparation when added in amounts of 0·1–0·4 ml. to the 15 ml. bath. The effect might be due to choline which is known to be present in small amounts in cerebrospinal fluid. The cerebrospinal fluid did not inactivate histamine as shown in experiments in which histamine was incubated with cerebrospinal fluid for 1 hr at 37° C.

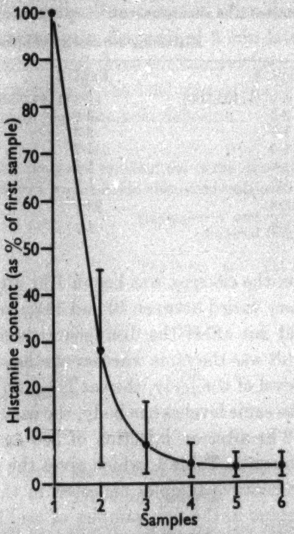

Fig. 5. Histamine recovery after intraventricular injection of 500 μg histamine in six successive samples collected from the cisterna magna in cats. Collection started 3 min after injection. The black dots give the mean values obtained in eight cats, the vertical lines the scatter of values.

Histamine. When 500 μg histamine was injected intraventricularly and 3 min later the collection of fluid from the cisterna was begun, between 40 and 77 % (mean 63 %) of the injected histamine was recovered. The first sample of undiluted c.s.f. collected from the cisternal cannula varied between 0·7 and 1·9 ml. and contained 27–56 % of the injected histamine. The histamine concentration in this sample varied between 1:3,000 and 1:11,000. The histamine content in the subsequent samples, each one collected during and after an intraventricular injection of 1 ml. of Tyrode solution to wash out the ventricular spaces, fell off steeply. This is illustrated in Fig. 5. It was not possible to increase the recovery by reducing the interval between injection and collection. In one experiment, for instance, in which collection was started 10 sec after the injection of 500 μg histamine, the recovery was 67 %. In another experiment the first sample of undiluted c.s.f. was collected 3 min after the injection of 500 μg of histamine in fourteen separate fractions of about 0·1 ml. each. The histamine concentration in the first three fractions was between 1:2300 and 1:2500 and then decreased quickly so that the concentration in the last four samples was about 1:20,000 only.

TABLE 1. Histamine content in successive samples of fluid collected from the cisterna magna in three cats. Collection started 2 hr after intraventricular injection of 500 μg of histamine

	Histamine content of samples (μg)		
Sample	Expt. 1	Expt. 2	Expt. 3
1	5·9 (1:102,000)	11·3 (1:44,000)	12·5 (1:16,000)
2	3·6	5·6	6·3
3	1·7	2·3	2·8
4	1·5	1·7	2·0
5	1·6	1·6	1·5
6	1·5	1·2	1·3
Total	15·8	23·7	26·4
Recovery (%)	3·2	4·7	5·3

When collection from the cisterna was begun 1 hr after injection of 500 μg of histamine, the recovery varied between 10 and 30 % (mean 20 %). Positional changes apparently did not affect the disappearance of histamine from the ventricles since the result was the same whether the head, fixed in the holder, was raised above the level of the body, the cat lying on its belly; or whether the head was kept at the same level as the body, the cat lying on its side. When collection was begun 2 hr after an injection of 500 μg histamine, the mean recovery was 4 % as shown in Table 1, which gives the amounts of histamine recovered in the six successive samples collected in three experiments. The concentration of histamine in the first samples of undiluted c.s.f. is given in brackets. When collection was begun 4 hr after an injection of 500 μg histamine, the recovery was 4·3 μg in the first, and 5·4 μg in the second experiment. The histamine content in the six consecutive samples of the second experiment was 1·9, 1·5, 0·7, 0·5, 0·4 and 0·4 μg. The flow from the cannula in the cisterna was only small and the 1·9 μg was obtained in 0·2 ml. c.s.f.

Acetylcholine. When collection was begun 3 min after an intraventricular injection of 500 μg acetylcholine, the recovery was of the same order as in the experiments with histamine. It varied between 42 and 67 % (mean 56 %) and the concentration in the first sample of undiluted c.s.f. collected from the cisterna varied between 1:2000 and 1:8000 (mean 1:3600). In one experiment collection was begun 1 hr after the injection of 500 μg acetylcholine together with 100 μg eserine. The recovery was 3·6 %. This is less than the recovery obtained in corresponding experiments with histamine.

Recovery after death. The finding that neither histamine nor acetylcholine could be fully recovered from the cisterna when the collection was begun 3 min after their intraventricular injection, or even earlier, raised the question whether this incomplete recovery was the result of factors pertaining to the living cat or whether it would occur in the dead cat as well. In order to compare the recovery from the living and the dead cat the following procedure was adopted. An intraventricular injection of 500 μg of either histamine or acetylcholine was made in the living cat and the recovery determined after 3 min. One hour later the cat was killed by an overdose of pentobarbitone administered intraperitoneally. Ten minutes after death, the injection was repeated and the recovery again determined 3 min later. The results of two experiments with histamine and three with acetylcholine are given in Table 2. In all of them recovery was less in the dead than in the living cat. The difference was greater for histamine than for acetylcholine.

Table 2. Comparison in the living and the dead cat of the recoveries of histamine and acetylcholine from the cisterna magna 3 min after intraventricular injection of 500 μg

	Histamine or acetylcholine (μg) recovered from	
	The living cat	The dead cat
Histamine	384	183
Histamine	355	133
Mean	370	158
Acetylcholine	276	243
Acetylcholine	275	235
Acetylcholine	210	164
Mean	254	214

Recovery in dogs of the injected histamine and acetylcholine from the cisterna

The results are given in Table 3. In all but the last experiment two injections were made. Collection after the first injection was 3 min later; the second injection was given after an interval of about 1 hr.

Three minutes after the intraventricular injection of either 500 μg of histamine or 500 μg of acetylcholine the recovery was between 49 and 77 %, whereas 1 hr after the injection the recovery varied between 3 and 12 %. There was no significant difference between the recoveries of histamine and of acetylcholine.

TABLE 3. Recovery of either histamine or acetylcholine from the cisterna magna of dogs after intraventricular injections. In each experiment 500 μg of the same drug was injected twice with an interval of 1 hr. Collection started 3 min after the first, and 30 or 60 min after the second injection

Expt. no.	Drug injected	Eserine* 100 μg	Percentage recovery of the drug after		
			3 min	30 min	60 min
1	Histamine	.	55	.	5
2	Histamine	.	63	.	3
3	Histamine	.	62	19	.
4	Acetylcholine	+	49	.	7
5	Acetylcholine	+	77	.	12
6	Acetylcholine	+	.	.	4

* Injection of eserine (100 μg) preceded the injection of acetylcholine by 7 min in Expts. 5 and 6, while it was given together with acetylcholine in Expt. 4.

Recovery of the injected histamine from the subarachnoidal space

From the lumbar subarachnoidal space in dogs and cats. Fig. 6 illustrates a typical experiment on a dog. Collection was started 1 hr after the injection of 500 μg of histamine. The first sample of 0·6 ml. c.s.f. contained no histamine, nor did the second sample of 1·1 ml. fluid collected when the flow which had stopped was restarted by an intraventricular injection of 1 ml. of Tyrode solution. However, histamine appeared in the subsequent five samples collected whilst 9 ml. of Tyrode solution was injected intraventricularly, first in increasing and then in decreasing concentrations. The total amount assayed was 32·8 μg. It is clear that no histamine had reached the lumbar region before the lumbar flow was started and that part of the 32·8 μg must have come from the ventricles, passing down to the lumbar subarachnoidal space with the fluid injected intraventricularly. Therefore in another experiment the following procedure was adopted. After the flow had ceased, the needle was closed, a cannula inserted into the cisterna magna and several samples of cisternal fluid were collected, whilst Tyrode solution was injected into the lateral ventricle in order to wash out the histamine present in the ventricles. The cisternal cannula was then closed, the needle in the lumbar region opened and the flow from the lumbar region restarted by intraventricular injections of Tyrode solution. This alternate collection from the lumbar and the cisternal regions was then repeated. The result, illustrated in Table 4, shows that in spite of washing the ventricles through the cisternal cannula, some histamine reached the lumbar subarachnoidal space when, after the end

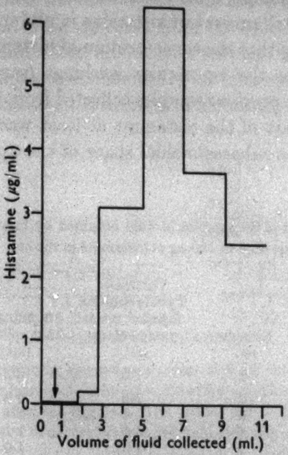

Fig. 6. Histamine content in successive samples of fluid obtained by lumbar puncture after an intraventricular injection of 500 µg of histamine into a 5·2 kg dog. Collection started 1 hr after the injection and continued for 35 min. From the arrow onwards Tyrode solution injected into the lateral ventricle during collection of each sample.

Table 4. Histamine content of the samples of fluid obtained by lumbar and cisternal punctures after intraventricular injection of 600 µg of histamine in the dog. Collection started 1 hr after the injection

Site of collection	Sample no.	Volume of Tyrode solution injected intra-ventricularly (ml.)	Volume of fluid collected (ml.)	Concentration of histamine in the collected fluid (µg/ml.)
Lumbar	1	Nil	0·6	Nil
	2	Nil	0·6	Nil
Cisternal	3	Nil	0·7	5·28
	4	2·0	1·0	2·40
	5	2·0	2·1	1·47
	6	2·0	2·0	1·50
	7	2·0	2·1	1·00
	8	1·0	1·7	0·76
	9	1·0	3·0	1·00
Lumbar	10	1·0	1·9	1·21
	11	1·0	0·5	1·80
Cisternal	12	1·0	1·3	0·22
	13	1·0	1·1	0·45
Lumbar	14	1·0	0·8	1·0
	15	1·0	0·4	0·75
	16	1·0	0·7	0·43
	17	1·0	0·8	0·41
	18	1·0	0·4	0·19
	19	1·0	1·2	0·13

of the ventricular washings, a flow was started from the ventricles to the lumbar region. The total amount of histamine in all lumbar samples collected was 5·2 μg. The finding that the concentration of histamine in the first lumbar samples collected after the ventricular washings (samples 10 and 14) was higher than that in the previous samples collected from the cisterna (samples 9 and 13) shows that part of the histamine at least was not coming from the ventricles but from the subarachnoidal space of the upper part of the spinal cord.

TABLE 5. Histamine content of the samples of fluid obtained by lumbar and cisternal punctures after intraventricular injection of 500 μg of histamine in the cat. Collection started 1 hr after the injection

Site of collection	Sample no.	Volume of Tyrode solution injected intraventricularly (ml.)	Volume of fluid collected (ml.)	Concentration of histamine in the collected fluid (μg/ml.)
Lumbar	1	Nil	0·2	1·1
Cisternal	2	Nil	0·9	60·0
	3	1·0	1·1	13·4
	4	1·0	1·1	6·1
	5	1·0	1·0	4·4
	6	1·0	1·0	2·9
	7	1·0	1·0	3·3
	8	1·0	1·0	2·85
Lumbar	9	1·0	0·2	8·3
	10	1·0	0·3	2·2
Cisternal	11	1·0	1·0	2·5
Lumbar	12	1·0	0·5	10·6
	13	1·0	0·45	11·8
Cisternal	14	1·0	1·1	0·45
	15	1·0	1·0	0·21
Lumbar	16	1·0	0·25	5·3
	17	2·0	0·15	1·73

In cats the first lumbar sample (0·2–0·3 ml.), collected 1 hr after an intraventricular injection of 500 μg, already contained 0·2–0·3 μg histamine; its concentration in subsequent samples collected during intraventricular injection of Tyrode solution rose and later fell again. Since part of this histamine must have come from the ventricles, the procedure of alternate collection from the lumbar and the cisterna regions was adopted. Such an experiment is illustrated in Table 5. The amounts of histamine collected from the lumbar region were higher than in dogs. This is not surprising, because the space of the ventricular system in cats is much smaller, yet the amount of histamine and the volume injected intraventricularly were the same in both species. The experiment shows that high concentrations of histamine must have reached the subarachnoidal space of the upper part of the spinal cord, because the concentration of histamine in the lumbar samples (samples 9, 12 and 16) was still high after it had fallen off in the cisternal samples (samples 8, 11 and 15).

From the epicerebral subarachnoidal space of the cat. When successive samples of about 0·1 ml. of c.s.f. were collected from the epicerebral subarachnoidal space starting 40–60 min after an intraventricular injection of 500 μg of histamine, the first sample again contained no histamine, or traces only. It appeared, however, in subsequent samples in increasing amounts. Two typical experiments are illustrated in Fig. 7. The fluctuations in histamine concentration of successive samples were obtained in all experiments. The total amounts of histamine recovered in both experiments were less than 4 μg. In one experiment in which collection was started 20 min after the intraventricular injection, the first sample also was free from histamine but its concentration in subsequent samples rose to over 1 μg/0·1 ml.; in this experiment 1·7 ml. was collected in thirteen successive samples containing a total of 14·2 μg of histamine.

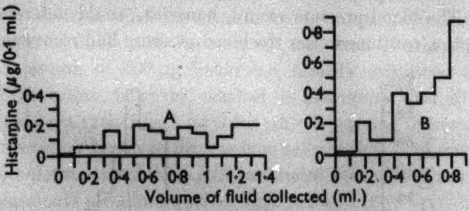

Fig. 7. Histamine concentration in successive samples of fluid obtained from the epicerebral subarachnoid space after intraventricular injection of 500 μg of histamine in two cats A and B. Collection started 1 hr after the injection. The dotted part indicates that the amounts of histamine were too small to be accurately assayed, probably they were smaller than indicated.

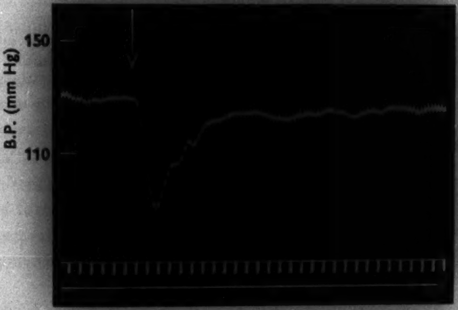

Fig. 8. Arterial blood pressure of a cat under chloralose anaesthesia. At the arrow (↓) intravenous infusion of histamine at a rate of 1·2 μg/min was begun and continued until the end of the tracing. Time marker, 30 sec.

Absorption into the blood stream in cats of intraventricularly injected histamine

The transient fall in arterial blood pressure which often followed the intraventricular injection of 500 μg of histamine in cats was attributed to an initial absorption of a small portion of the injected histamine into the blood stream (see p. 171). The blood-pressure record, however, would not reveal whether or not absorption continued after the blood pressure had recovered, because a

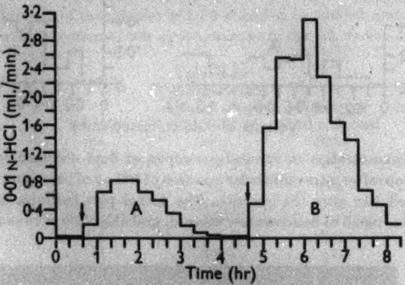

Fig. 9. Acid gastric secretion in a cat under chloralose anaesthesia with the intraventricular cannula implanted 2 weeks before the experiment. Comparison of the secretion in response to an intraventricular injection of 500 μg of histamine (at A) with that in response to a subcutaneous injection of 500 μg of histamine (at B); injections indicated by arrows.

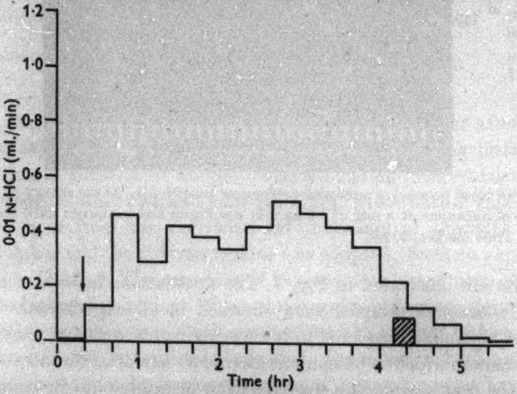

Fig. 10. Acid gastric secretion in response to an intraventricular injection of 500 μg of histamine in a vagotomized cat under chloralose anaesthesia. The intraventricular cannula had been implanted 1 week before the experiment. Time of injection indicated by the arrow (↓). Shaded area indicates washing out of histamine from the ventricles.

continuous intravenous infusion of histamine at a rate of about 1 μg/min also leads to a transient depressor effect only, as shown in the experiment of Fig. 8. The depressor effect resembles closely that seen in experiment Fig. 2C after an intraventricular injection of 500 μg of histamine. A slow continuous absorption of histamine into the blood stream, however, is easily detected by the acid gastric secretion.

Acid gastric secretion after intraventricular injection of histamine. Usually the first sample of fluid from the stomach, collected 20 min after an intraventricular injection of 500 μg histamine, already contained some hydrochloric acid. Secretion, however, reached its maximum after 40–80 min and then continued on a rather high level for periods varying between 1 and over 3 hr. When secretion began to decline it usually took 2–3 hr until it came to an end. To accelerate this decline, the ventricles were sometimes washed out. Typical experiments are illustrated in Figs. 9A and 10.

The secretion was not the result of a central action of the injected histamine either via the vagi or via the extravagal pathway postulated by Porter, Movius & French (1953). According to these authors stimulation of the posterior hypothalamus may lead to acid gastric secretion by release of ACTH, which would stimulate the suprarenals to release cortisone which causes acid gastric secretion as shown by French, Longmire, Porter & Movius (1953). Since the acid gastric secretion obtained by intraventricular injection of histamine occurred also after section of both vagi in the neck (see Fig. 10) and after removal of the suprarenals, the vagal and extravagal central secretory pathways have been excluded. The secretion must thus be a response of the oxyntic cells to the absorbed histamine.

Since the secretion was also obtained in experiments in which the cannula used for the intraventricular injection had been implanted 1–2 weeks before the actual experiment was performed, as in the experiments (Figs. 9, 10 and 13), the absorption was not from brain tissue freshly injured on insertion of the cannula.

In order to obtain more quantitative data about the rate of absorption, the acid gastric secretion produced by intraventricular injection of histamine was compared with that of histamine injected subcutaneously or slowly infused intravenously. For such a comparison the following facts have to be taken into account.

(1) When the same amount of histamine is given a second or third time, the acid gastric secretion increases with each administration. This happens whether the histamine is injected intraventricularly or subcutaneously, or infused intravenously. This is illustrated for two subcutaneous injections in Fig. 11.

(2) The secretion evoked by a slow intravenous infusion of histamine does not come to an end immediately when infusion stops, but continues for up to

1 hr at a relatively low level. Therefore, the end point of secretion after a subcutaneous or intraventricular injection of histamine does not coincide with the end point of absorption, and it is not possible in these circumstances to say for certain what part of the 'tail end' of secretion is still a sign of absorption.

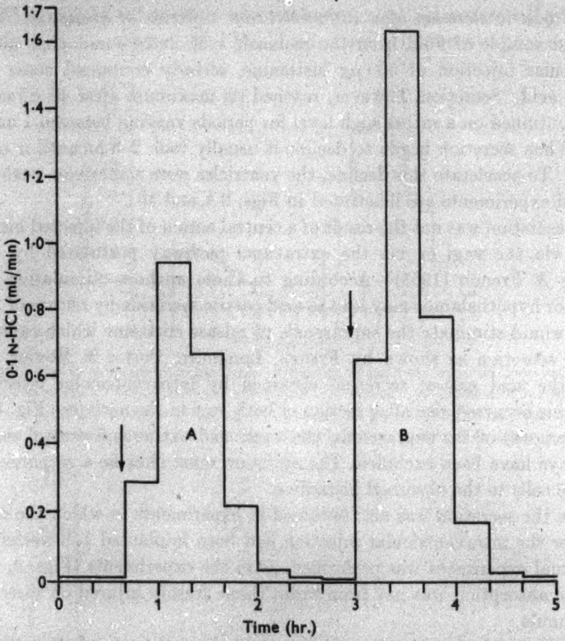

Fig. 11. Acid gastric secretion resulting from two successive subcutaneous injections of 250 μg histamine (at A and B) in a cat under chloralose anaesthesia. Injections indicated by arrows.

(3) If histamine is infused intravenously at a constant rate, the acid secreted increases in each successive 20 min sample. No definite peak of secretion was reached in these experiments even when infusion was continued for 4 hr. This means that when a peak of secretion is reached after an intraventricular injection of histamine, the rate of absorption may have been maximal some time before the peak was reached.

The maximal rate of secretion after an intraventricular injection of 500 μg of histamine was found to be less than that obtained after a subcutaneous injection of 500 (Fig. 9) and even of 250 μg of histamine, whether the subcutaneous injection preceded or followed the intraventricular injection. This suggests that the rate of absorption after a subcutaneous injection of histamine is at least twice as great as after an intraventricular injection.

In several cats the maximal rate of acid gastric secretion in response to an intraventricular injection of 500 μg of histamine was compared with that obtained in response to slow intravenous infusions for 1 hr. The amounts of histamine infused per minute varied between 0·6 and 1·8 μg. Fig. 12 illustrates an experiment in which the acid secretory response to an intraventricular injection of 500 μg of histamine was bracketed between the responses to two intravenous infusions of 0·6 μg histamine per minute. The secretion induced by the intraventricular injection reached a higher value irrespective of whether it followed or preceded the infusion. Therefore the maximal rate of absorption after intraventricular injection must be greater than 0·6 μg/min. Fig. 13 illustrates experiments on two cats in which the acid gastric secretion in response to an intravenous infusion of either 1·8 μg or 1·4 μg/min histamine reached higher levels than in response to a subsequent intraventricular injection of 500 μg of histamine. Thus the maximal rate of absorption after an

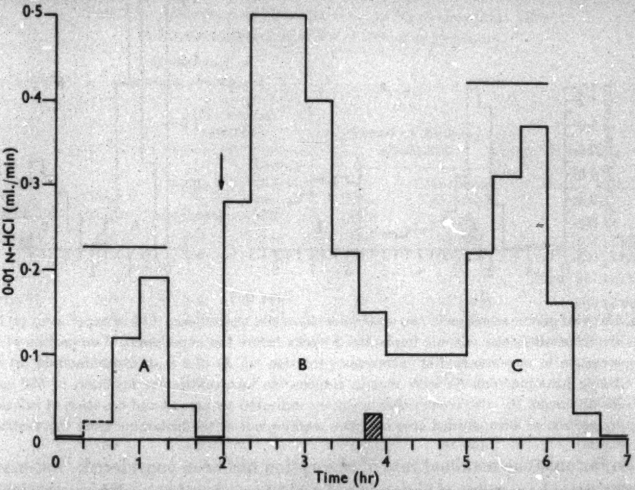

Fig. 12. Acid gastric secretion in a cat under chloralose anaesthesia. Comparison of the secretion in response to intravenous infusions of histamine 0·6 μg/min for 1 hr (at A and C) with that in response to intraventricular injection of 500 μg of histamine (at B). Time at which the intraventricular injection was given is indicated by an arrow and the duration of infusions by horizontal lines. Dotted part in A signifies that the acid secretion was probably less than indicated; shaded area indicates washing out of histamine from the ventricle.

intraventricular injection of 500 μg histamine must have been less than 1·8 and 1·4 μg/min. In further experiments it was found that the maximal secretion produced by an infusion of 1·2 μg/min was equal or only slightly greater than that obtained after an intraventricular injection of 500 μg histamine. Thus the maximal rate of absorption of the intraventricular injection of 500 μg of histamine corresponded to an absorption of 1·2 μg histamine/min or slightly less.

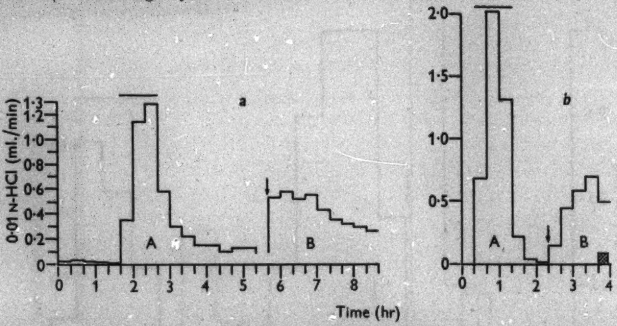

Fig. 13. Acid gastric secretion in two cats under chloralose anaesthesia. Cat of experiment (*a*) had the intraventricular cannula implanted 2 weeks before the experiment. Comparison of the secretion in response to 1 hr intravenous infusion (at A) of 1·8 μg histamine/min (*a*) and 1·4 μg histamine/min (*b*) with that in response to intraventricular injection of 500 μg of histamine (at B). Intraventricular injections indicated by arrows and duration of infusions by horizontal lines; shaded area indicates washing out of the histamine from the ventricle.

So far only the maximal rate of absorption has been considered. Estimates of the actual amounts of histamine absorbed can be obtained from the histograms of secretion by assuming that the maximal rate at which histamine enters the blood stream in the initial stages of increasing secretion is either 1·2 μg/min or 1 μg/min and that, from the moment there is a decline in the secretion, the rate of histamine entry into the blood stream diminishes gradually to 0. The period of maximal absorption is easily obtained from the histogram. The calculation for the absorption during the declining phase of secretion varies, however, according to whether the end point of absorption is taken to coincide with the end of secretion, or to have taken place one hour earlier, and according to whether one assumes a linear or an exponential decline of absorption. The values obtained for an exponential decline during the period of diminishing absorption are about 30% less than for a linear decline.

In Table 6 calculations have been made for five experiments. For each experiment two calculations are made. One is based on a maximal absorption of 1·2 μg/min and on the assumption that the end point of absorption coincides with the end of secretion. This calculation errs on the high side. The other calculation is based on a maximal absorption of 1·0 μg/min and on the assumption that the end point of absorption occurs 1 hr before the end of secretion. This calculation errs on the low side. Both calculations are based on a linear decline of absorption; an exponential decline would reduce the values for the declining phase by about 30 % and these reduced values are given in brackets in the table as well.

TABLE 6. Absorption of histamine into the blood circulation after an intraventricular injection of 500 μg of histamine

	Initial period of maximal absorption			Period of declining absorption		
		Histamine absorbed				
Expt. of	Time (min)	(μg/min)	During initial period (μg)	Time (min)	Histamine absorbed (μg)	Histamine absorbed during both periods (μg)
Fig. 14	140	1·2	168	160	95 (66)	263 (234)
	140	1·0	140	100	50 (35)	190 (175)
						Mean 227 (205)
Fig. 10	40	1·2	48	260	157 (110)	205 (158)
	40	1·0	40	200	100 (70)	140 (110)
						Mean 173 (134)
	60	1·2	72	120	72 (50)	144 (122)
	60	1·0	60	60	30 (21)	90 (81)
						Mean 117 (102)
Fig. 13*a*	40	1·2	48	220	132 (92)	180 (140)
	40	1·0	40	160	80 (56)	120 (96)
						Mean 150 (118)
Fig. 12	40	1·2	48	140	84 (59)	132 (107)
	40	1·0	40	80	40 (28)	80 (68)
						Mean 106 (88)

In the first experiment of Table 6 the period of increasing gastric secretion lasted for 140 min. This was taken as the period of maximal absorption. Thus 168 or 140 μg would be absorbed at a rate of 1·2 or 1 μg/min respectively. The acid gastric secretion came to an end after a further 160 min so that for the end point of absorption either 160 or 100 min are taken. The method of calculation of the amounts absorbed during these 100 or 160 min periods is shown in Fig. 14. On the left, (A), the calculation is based on the assumption that at the beginning of the declining phase of absorption the rate was 1·2 μg/min and that the decline proceeded in a linear manner to 0 in 160 min. On the right, (B), the calculation is based on the assumption that at the beginning of the declining phase of absorption, the rate was 1 μg/min and that the decline proceeded in a linear manner to 0 in 100 min. The straight oblique lines show the decline in absorption values to zero in 160 or 100 min from the initial values of either 1·2 or 1·0 μg/min respectively. By taking the points where the oblique line cuts the 10, 30, 50, 70, 90, 110, 130 and 150 min periods, the mean rate of absorption per minute for each successive 20 min sample is obtained. This rate is given beside the oblique lines and the figures represent μg/min. Further, by multiplying these values by 20, the μg of histamine entering the circulation during each successive 20 min sample are obtained (figures in brackets). By adding these amounts together, the total absorption expressed in μg histamine during the declining phase is obtained. This value is given in column 6 of Table 6. In the same way the values for the other experiments of Table 6 have been calculated. If we take the mean values of the two calculations for each of the five experiments of Table 6, between 21 and 45% of the injected histamine would have entered the circulation, or between 18 and 41% when the calculation during the declining phase is based on an exponential decline of absorption.

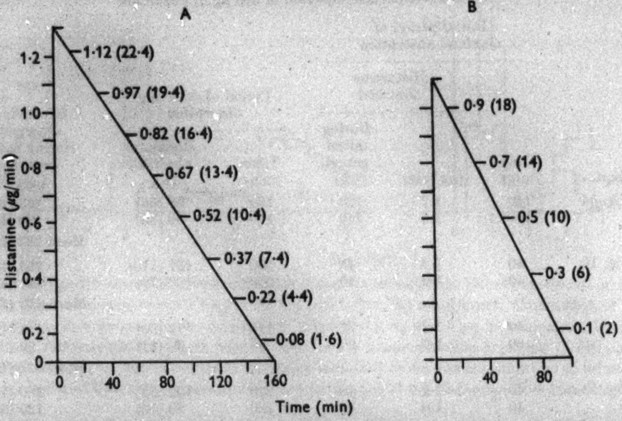

Fig. 14. Illustration of the calculation (for the first experiment Table 6) used for determining the amounts of histamine absorbed during the declining phase of acid gastric secretion, assuming linear decline. A: based on a maximal absorption of 1·2 μg/min and a declining phase lasting for 160 min. B: based on a maximal absorption of 1·0 μg/min and a declining phase lasting for 100 min. (For details, see text.)

DISCUSSION

When 500 μg histamine or acetylcholine was injected into the lateral cerebral ventricle of the cat, and c.s.f. was collected from the cisterna magna—the collection being started 3 min or even earlier after the injection, and the ventricle washed during the collection—the injected histamine or acetylcholine was not fully recovered. In fact, with this method, the recovery was only 40–77 % or, on the average, about two-thirds of the injected histamine.

The problem arises as to why the recovery was not complete and where the remaining part of the histamine had gone. It is possible that the volume used for making the injection (0·25 + 0·25 ml.) pushed the histamine through the ventricular system, not only to the cisterna magna but also to parts beyond this region of the subarachnoidal space. When the collection from the cisterna magna was made 3 min after the injection in successive fractions of 3 drops each, the first fraction contained histamine in a concentration of about 1:2300. Even if the last drop of this fraction were already contaminated with the ventricular fluid, it is certain that, 3 min after the injection, the c.s.f. in the cisterna magna contained a high concentration of histamine. Since the cisterna magna communicates freely with the regions of the subarachnoidal space surrounding the base of the brain and upper part of the cervical cord, it may be assumed that some of the unrecovered histamine had diffused into these regions and escaped recovery by cisternal puncture and subsequent washing of the ventricle. The experiments on acid gastric secretion show that histamine reaches the general blood circulation within the first 20 min after intraventricular injection and must thus have traversed a greater distance of the subarachnoidal space than that adjoining the cisterna.

The finding that the recovery was even less in the dead than in the living cat could be explained by a greater escape of the injected drug into parts of the subarachnoidal space adjoining the cisterna magna. The volume of the cranial box does not change, nor does that of the brain. The brain, however, does not occupy the whole of the cranial box. The remaining space is taken up by the c.s.f. and the blood vessels. During dilatation of the vessels the subarachnoidal space taken up by the c.s.f. decreases, and during contraction it increases. Thus in order to understand why after death more of the injected histamine escapes into the regions of the subarachnoidal space not drained by the method used for collection from the cisterna magna, it is only necessary to assume that after death there is a reduction in the volume taken up by the vessels and consequently a relative increase in the space occupied by the c.s.f.

The experiments on acid gastric secretion after intraventricular injection of histamine show that part of the injected histamine is absorbed into the blood stream. When the absorption of histamine into the blood stream is correlated with its disappearance from the cerebral ventricle, it is found that, during the first hour after an intraventricular injection the rate of absorption is greatest and so is the rate of disappearance. The time course of maximal absorption also appears to parallel the maximal rate of disappearance of histamine from the ventricles, particularly if we take into account that the injected histamine has to traverse a relatively large area of the subarachnoidal space before it is absorbed into the endocranial venous sinuses. However, the parallelism breaks down when the actual amounts disappearing from the ventricles and those being absorbed into the blood stream are correlated, because about 80% disappears during the first hour and about 99% within 4 hr, whereas the absorption as calculated from the total period of acid gastric secretion accounts for less than half of the injected histamine only. About the fate of that part of histamine which is not accounted for by absorption into the blood stream it is at present possible only to speculate. A certain amount is certainly accounted for by the histamine reaching the subarachnoidal space around the brain stem and upper spinal cord from where it would be absorbed only very slowly, but part of the unrecovered histamine may have been taken up by the brain substance or may have been destroyed enzymatically on its way to the venous sinuses. This pertinent problem has not been examined.

One of the reasons for performing the experiments presented in the present paper was to find out whether the long-lasting effects observed in unanaesthetized animals after intraventricular injection of the drugs were associated with the persistence of the drugs in the ventricles. Feldberg & Sherwood (1954) found that within an hour after an intraventricular injection of 150–200 μg of histamine, in cats, the main effects became weaker and the recovery, though not complete, started within this period. In the present experiments it was found that 1 hr after the intraventricular injection of 500 μg of histamine about 20% of the histamine could still be recovered. There is thus, as far as histamine is concerned, no reason to assume that the action it produces persists after the histamine has left the ventricular spaces.

SUMMARY

1. In anaesthetized cats and dogs histamine or acetylcholine was injected into the cerebral lateral ventricle and, at various times after the injection, the cerebrospinal fluid was removed from the cisterna magna, the ventricular spaces were washed out by repeated ventricular injections, the washings were also collected and the samples assayed for histamine or acetylcholine respectively.

2. In cats the mean recoveries of histamine from the cisterna were 63% when collection was started 3 min, 20% when started 1 hr, and 4 and 1% when started 2 and 4 hr respectively after the injection. In cats acetylcholine disappeared earlier from the ventricles since only a small percentage could be recovered from the cisterna 1 hr after the injection.

3. In dogs the recoveries of histamine or acetylcholine from the cisterna were between 49 and 77% when collection was started 3 min, and between 3 and 12% when collection was started 1 hr after the injection.

4. One hour after an intraventricular injection of 500 μg of histamine none, or only small amounts, were detected in the c.s.f. from the lumbar or epicerebral subarachnoidal spaces.

5. The intraventricular injection of 500 μg of histamine into the anaesthetized cat caused acid gastric secretion, which could be attributed to absorption of the histamine into the blood stream.

6. By comparing the acid gastric secretion with that of a slow intravenous infusion of histamine the maximal rate of absorption after an intraventricular injection of 500 μg of histamine was found to be between 1 and 1·2 μg/min.

7. From the histograms of acid gastric secretion the amounts of histamine absorbed into the blood stream were calculated to be less than half the amount injected intraventricularly. Part of the unaccounted histamine might be the histamine lingering in the subarachnoidal spaces around the brain stem and upper spinal cord from where it would be absorbed slowly.

I am grateful to Sir Charles Harington for the opportunity of working in the National Institute for Medical Research, and to Dr W. Feldberg for his advice and encouragement.

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THE EFFECT OF ALTERATIONS OF PLASMA SODIUM ON THE SODIUM AND POTASSIUM CONTENT OF MUSCLE IN THE RAT

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It is now generally accepted that the unequal distribution of ions between cells and extracellular fluid is maintained by metabolic activity of the cells, the low cell sodium content in particular being explained by a mechanism of active sodium extrusion (Dean 1941). Although many studies have been made *in vitro* on the effect of altered environment upon muscle electrolytes (Harris, 1950; Steinbach, 1952), and upon the mechanism of sodium extrusion in various tissues (Hodgkin & Keynes, 1954; Carey & Conway, 1954; Edwards & Harris, 1957), comparatively little is known as to the response of the sodium extrusion mechanism of muscle *in vivo* when the external sodium concentration is varied extensively.

In the present study acute alterations in plasma sodium were produced by water and salt loading in the conscious rat, and the effects of these procedures upon the intracellular content of water, sodium and potassium in cardiac and skeletal muscle were determined, both in normal and adrenalectomized animals.

METHODS

The principles involved in the calculation of the intracellular concentration of an ion have been given by Boyle & Conway (1941), and by Manery (1954). Muscle water is considered to exist in two phases, namely intracellular and extracellular or interstitial water. In the present experiments inulin space was taken as a measure of extracellular water. Intracellular water is determined by deducting the extracellular water from the total water content of the tissue. The amount of any ion in the intracellular phase can similarly be calculated by deducting from the total amount of tissue ion the amount contained in a known volume of extracellular water. The intracellular concentration is obtained by correcting the intracellular ion to the amount that would occupy 1 l. of cell water.

Operative procedure. Wistar strain albino rats of 200–250 g body weight were used. The method used for determining inulin space was that described by Ledingham (1953). Rats of known weight were lightly anaesthetized with ether, both renal pedicles ligated through a dorsal incision, and an inulin solution (22 %, w/v) was injected into an external jugular vein, in the dose of 0·5 ml./100 g body weight. The animals were deprived of food and water, and after 6 hr blood was collected from a carotid artery after the administration of heparin (Liquemin, Roche, Products, 500 u./kg body wt.). The heart was then excised, the great vessels, auricles and right ventricle cut away and the left ventricle cut into strips. Surface moisture was removed by the gentle application of blotting paper. Specimens were then placed in tubes of known weight for the determinations described below. The gastrocnemius was used for skeletal muscle determinations. After careful removal of fascia and nerves, the belly of the muscle was similarly divided into pieces, blotted and placed in tubes of known weight.

Muscle water. Muscle specimens of 50–80 mg wet weight were weighed to within 0·1 mg, dried for 24 hr at 100° C, and then re-weighed after cooling to constant weight. Total water content was calculated from the weight loss, in terms of ml./kg wet wt. of tissue. At least two specimens of each type of muscle were used in each experiment.

Muscle inulin. Muscle specimens of 150–250 mg wet weight were weighed to within 0·1 mg in stoppered tubes. Inulin in muscle was determined by the method of Ross & Mokotoff (1951). The method provides for a muscle blank with each experiment and one specimen each of cardiac and of skeletal muscle was used for this purpose.

Plasma inulin was determined by the method of Roe, Epstein & Goldstein (1949). The inulin concentration of extracellular water was taken as 1·1 × plasma inulin (Ledingham, 1954). Extracellular water was calculated from the muscle and plasma inulin, and expressed in ml./kg wet wt. of muscle.

Muscle Na and K. The muscle specimens used for estimation of total water content were dissolved in 1 ml. 50% (w/v) nitric acid in a boiling water-bath. The solutions were diluted appropriately with ion-free distilled water and Na and K estimated by flame photometry. Results are expressed in m-equiv/kg wet wt. of tissue.

Plasma Na and K were measured by flame photometry after appropriate dilution. The concentration of Na in the extracellular fluid is taken as 0·96 × plasma Na to allow for the Donnan effect.

Adrenalectomy. Bilateral adrenalectomy was carried out through a dorsal incision under light ether anaesthesia. The rats were then maintained on a standard cube diet with 0·5% (w/v) NaCl as drinking water for 3 days before experiment.

Water and salt loading. Water loading was effected by an intraperitoneal injection of distilled water (12·5 ml./100 g body wt.) and salt loading by intraperitoneal injection of a 5% (w/v) solution of NaCl. These volumes were given 3 hr after the inulin injection. Inulin equilibrates in cardiac and skeletal muscle in 3 hr (Ledingham, 1953) and it was considered that had a large volume of intraperitoneal water or saline been given at the same time as the inulin delay in equilibration might occur. The animals were killed 3 hr after the administration of water or saline.

RESULTS

In a preliminary investigation, carried out to see if the presence of a large volume of intraperitoneal fluid could affect the measurement of inulin space, four rats were given an intraperitoneal injection of normal saline solution (0·9%, w/v) in the dose 12·5 ml./100 g body wt., 3 hr after intravenous inulin. After a further period of 3 hr, blood and tissues (heart and skeletal muscle) were collected and inulin space determined. The mean value (± S.E.) obtained for cardiac muscle was 120 ± 8 ml./kg wet wt. and for skeletal muscle 60 ± 6 ml./kg wet wt. These values were not significantly different from those obtained in a control series of nine experiments in which rats were left for 6 hr after the inulin infusion, the inulin spaces being: cardiac muscle 112 ± 8·1 ml./kg wet wt. and skeletal muscle 55 ± 6·7 ml./kg wet wt. It was evident that the presence of a large volume of intraperitoneal fluid did not affect the measurement of inulin space.

The 3 hr period allowed for the equilibration of the injected water or salt load was also considered adequate. In four experiments (two using water and two using saline) blood and tissues were collected 5 hr after intraperitoneal injection of the water or saline. The values obtained for plasma and total muscle Na and K did not differ from the values obtained using the shorter period of 3 hr.

The effect of water loading on muscle water and electrolytes

The effect of water loading on muscle water and electrolyte was determined in six experiments. Rats were lightly anaesthetized with ether, both renal pedicles ligated and intravenous inulin given. After a 3 hr equilibration period an intraperitoneal injection of water (12·5 ml./100 g body wt.) was given. A further 3 hr period was allowed and the blood was collected from a carotid artery and tissues removed for analysis. The absorption of water from the peritoneal cavity in these experiments was satisfactory, the residual water not being greater than 2·5 ml./100 g body wt. in any experiment. The results given below are compared in each case with nine controls, in which tissues were obtained for analysis 6 hr after inulin injection.

Plasma Na and K. The mean plasma Na in the water-loaded group was 109 ± 1·5 and that in the control group 147 ± 1·0 m-equiv/l., the lowering of plasma Na being highly significant ($P < 0{\cdot}001$). The mean plasma K in the water-loaded group was 4·8 ± 0·5 m-equiv/l., a figure not significantly different from the mean value of 5·3 ± 0·1 m-equiv/l. obtained in the control group.

Muscle water. Values for total water, inulin space and intracellular water for cardiac and skeletal muscle in the water-loaded and control groups are shown in Table 1. It can be seen that in both types of muscle the total water content increased. With cardiac muscle there was a decrease in the inulin space and an increase in intracellular water amounting to 7·9 % of the initial cell water. The inulin space in skeletal muscle did not differ in the two groups and the increase in intracellular water amounted to 2·1 % of the initial water.

Muscle Na. The values for total muscle Na and the extracellular and intracellular Na fractions for cardiac muscle are shown in Fig. 1*A* and for skeletal muscle in Fig. 1*B*. From Fig. 1*A* it can be seen that with cardiac muscle there was a fall both in total muscle Na and in the extracellular fraction. The intracellular Na however remained unchanged. The results with skeletal muscle differed from those with cardiac muscle. From Fig. 1*B* it can be seen that there was a fall in total Na, a small decrease in extracellular Na and a highly significant decrease in intracellular Na.

The intracellular concentration of Na in cardiac muscle, calculated from the intracellular Na fraction (Fig. 1*A*) and the total water content (Table 1), decreased slightly as a result of the increase in cell water; the mean value of 23·8 ± 0·8 m-equiv/l. of cell water was not, however, significantly different from the control value of 25·1 ± 1·8 m-equiv/l. of cell water ($P < 0{\cdot}7$). With skeletal muscle the mean value for intracellular Na concentration of 6·8 ± 1·0 m-equiv/l. of cell water was significantly lower than the control value of 15·6 ± 1·6 m-equiv/l. of cell water ($P < 0{\cdot}01$), the result of a large loss of intracellular Na and a small increase in cell water.

Muscle K. Fig. 2*A* shows the total K content for cardiac and skeletal muscle in the water-loaded and control groups. The extracellular K fraction is less than 1 % of the total tissue K. It is therefore not represented in the diagram and intracellular K may be regarded as similar to total K. With cardiac

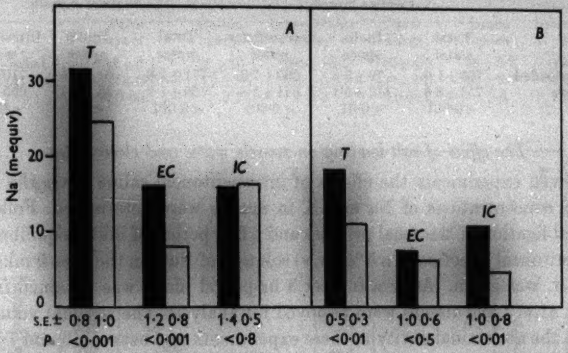

Fig. 1. The effect of water loading on the Na content of (*A*) cardiac muscle and (*B*) skeletal muscle. Black columns controls, open columns water-loaded. *T* total Na; *EC*, extracellular Na; *IC*, intracellular Na (m-equiv/kg wet wt.).

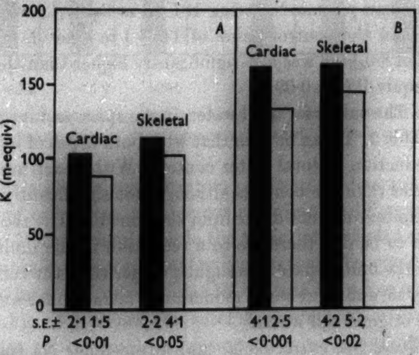

Fig. 2. The effect of water loading on the K content of cardiac and skeletal muscle. (*A*) Total K/kg wet wt. (*B*) K concn./l. cell water. Black columns controls, open columns water-loaded.

muscle the loss of K is significant ($P < 0{\cdot}01$): with skeletal muscle the results were less clear. The differences in intracellular concentrations of K, calculated as already described, are shown in Fig. 2*B*. The loss of K from cardiac muscle (Fig. 2*A*) and in the increase of cell water (Table 1), resulted in a marked lowering of intracellular concentration. With skeletal muscle the small increase in cell water further lowered the K concentration to a level which was probably significant.

TABLE 1. Total water content, inulin space and intracellular water of cardiac and skeletal muscle with water loading (ml./kg wet wt.)

	Cardiac muscle			Skeletal muscle		
	Total water	Inulin space	Intracellular water	Total water	Inulin space	Intracellular water
Water-loaded	769 ± 1·0	79 ± 9·8	690 ± 7·3	771 ± 2·6	61 ± 4·5	710 ± 3·1
Controls	753 ± 2·8	112 ± 8·1	641 ± 5·8	750 ± 1·9	55 ± 6·7	695 ± 6·8
P	< 0·001	< 0·01	< 0·001	< 0·001	< 0·6	< 0·001

The effect of salt loading on muscle water and electrolytes

In seven experiments the effects of intraperitoneal saline upon the intracellular concentrations of Na and K in muscle were determined. Following bilateral ligation of the renal pedicles and a 3 hr period of inulin equilibration, intraperitoneal injection of a 5 % (w/v) solution of NaCl in the dose 5 ml./100 g body wt. was given. After a further 3 hr period blood was obtained from a carotid artery and tissues were removed for analysis. The residual volume of fluid in the abdominal cavity in these experiments was between 6 and 7·5 ml./100 g body wt. The results given below are compared with nine controls in each case.

Plasma Na and K. A considerable rise in plasma Na resulted from this procedure, the mean plasma Na being 187 ± 6 m-equiv/l., which was significantly higher than the control value of 147 ± 1 m-equiv/l. ($P < 0{\cdot}001$). The plasma K level of 8·0 ± 0·8 was also significantly higher than the control value of 5·3 ± 0·1 m-equiv/l. ($P < 0{\cdot}001$).

Muscle water. The values for total water, inulin space and intracellular water are shown in Table 2. It can be seen that with both types of muscle there was a significant reduction in total water content. With heart muscle the inulin space was smaller than the controls although not significantly so. The intracellular water content did not differ from the controls. The reduction of total water appeared to be due therefore to a reduction in the inulin space. With skeletal muscle the inulin space was slightly larger than the controls although not significantly so, and there was a reduction of intracellular water amounting to 6·5 % of the original cell water.

Muscle Na. Total Na, extracellular and intracellular Na fractions for cardiac muscle are shown in fig. 3*A* and for skeletal muscle in Fig. 3*B*. It can be seen that with cardiac muscle there were small increases in each Na fraction, but that these increases were not significant. The absence of a significant rise in total sodium in spite of high extracellular Na concentration was due to the smaller inulin space found in this group. It was evident, however, that no large increase in the intracellular Na had taken place. With skeletal muscle, however, there was a large increase in total Na and a highly significant increase in the intracellular Na.

Table 2. Total water content, inulin space and intracellular water of cardiac and skeletal muscle with salt loading (ml./kg wet wt.)

	Cardiac muscle			Skeletal muscle		
	Total water	Inulin space	Intracellular water	Total water	Inulin space	Intracellular Water
Salt-loaded	763±3·1	92±10·1	644±10·3	716±4·5	66±8·8	650±9·3
Controls	753±2·8	112±8·1	651±5·8	750±1·9	55±6·7	695±6·8
P	<0·001	<0·1	<0·2	<0·001	<0·4	<0·001

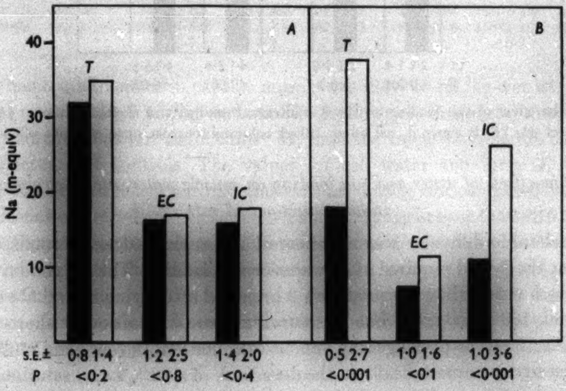

Fig. 3. The effect of salt loading on Na content of (*A*) cardiac and (*B*) skeletal muscle. Black columns controls, open columns salt-loaded. Symbols as for Fig. 1.

The mean intracellular concentration of Na in cardiac muscle was 29·3 ± 3·0 m-equiv/l. of cell water, a value not significantly different from the controls ($P < 0·3$). With skeletal muscle the mean intracellular concentration of 39·8 ± 5·6 m-equiv/l. was significantly higher than the controls ($P < 0·001$), the result of an increased intracellular Na content and a loss of cell water.

Muscle K. Fig. 4*A* shows the total K content of cardiac and skeletal muscle and Fig. 4*B* the final concentrations in m-equiv/l. of cell water. It can be seen that with cardiac muscle there was a large increase in the total K content, and the final intracellular concentration was also significantly higher than the controls. The very small increase in total K in skeletal muscle was not significant. The probably significant increase in final concentration of K is due therefore to the loss of cell water.

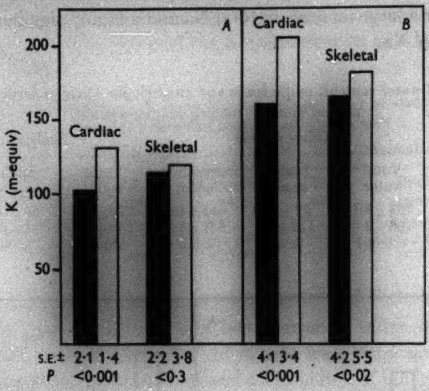

Fig. 4. The effect of salt loading on the K content of cardiac and skeletal muscle. (*A*) Total K/kg wet wt. (*B*) K concn./l. cell water. Black columns controls, open columns salt-loaded.

The effects of water and salt loading on muscle water and electrolytes in adrenalectomized rats

Considerable difficulty was experienced in keeping adrenalectomized rats alive for the period required after water or salt loading. The maximum water load which would allow survival for a 3 hr period after tying the renal pedicles was 7 ml./100 g body wt., but this survival time was seriously shortened if operation time was prolonged by venous cannulation. Even when inulin was administered intraperitoneally in the dose 1 ml. of a 22% (w/v) solution/100 g body wt., a 3 hr period allowed for equilibration, and then a further 3 hr allowed after water loading with the reduced volume, it was found possible to obtain complete data only in three experiments out of a large series. With salt loading complete data were obtained in five experiments by using a reduced volume of 2·5 ml. of a 5% (w/v) solution of NaCl following intraperitoneal inulin.

Blood inulin levels obtained after intraperitoneal inulin were as high as those obtained by the smaller dose intravenously, and sometimes higher. In three experiments using adrenalectomized rats the values for inulin space obtained after intraperitoneal inulin were of the same order as those obtained in a control series given inulin intravenously. A 6 hr equilibration period was allowed in each case.

The effect of adrenalectomy on muscle water and electrolytes. In six rats, used as a control series, muscle water, Na and K were determined 3 days after adrenalectomy. In these experiments an intravenous injection of inulin was given after ligation of the renal pedicles and blood and tissues were collected for analysis after 6 hr. The results given below are compared with nine non-adrenalectomized controls in each case.

The plasma Na concentration in the adrenalectomized group was 137 ± 1 m-equiv/l. and the plasma K 6·4 ± 0·52 m-equiv/l. These values were significantly different ($P < 0{\cdot}01$) from the non-adrenalectomized control series.

TABLE 3. The cell water, intracellular Na and K concentrations in adrenalectomized rats compared with non-adrenalectomized controls

	Cardiac muscle			Skeletal muscle		
	Cell water (ml./kg)	Na (m-equiv/l.)	K (m-equiv/l.)	Cell water (ml./kg)	Na (m-equiv/l.)	K (m-equiv/l.)
Adrenalectomized	646 ± 12	28·8 ± 3·0	154 ± 5·8	717 ± 9·5	14·7 ± 1·5	142 ± 2·9
Controls	641 ± 5·8	25·1 ± 1·8	162 ± 4·1	695 ± 6·8	15·6 ± 1·6	165 ± 4·2
P	< 0·7	< 0·2	< 0·2	< 0·02	< 0·7	< 0·01

The mean inulin space in cardiac muscle was 119 ± 10 ml./kg wet wt. and in skeletal muscle 43 ± 7 ml./kg wet wt. The inulin space in skeletal muscle was slightly smaller, but not significantly so, than the values obtained in the non-adrenalectomized controls. The values for cell water and intracellular Na and K concentrations in cardiac and skeletal muscle are compared with the non-adrenalectomized control series in Table 3. It can be seen that with cardiac muscle there were no significant differences from the control series in any of these values. With skeletal muscle there was an increase in cell water which was possibly significant, no change in Na concentration, but a significant lowering of intracellular K concentration. The lowering of K concentration was due to both an increase in cell water and a loss of K from the muscle, since the mean values of total K content were: non-adrenalectomized controls 115 ± 2·2 m-equiv/kg wet wt., adrenalectomized controls 102 ± 1·5 m-equiv/kg. wet wt. These values are significantly different ($P < 0{\cdot}001$).

The effect of water loading. The results given below, and compared with adrenalectomized controls in each case, represent complete data from three experiments in which values for inulin space were obtained, and from three other experiments in which the renal pedicles were ligated and tissues removed for Na and K estimation 3 hr after water loading. Plasma Na was significantly lowered to a mean value of 100 ± 6 m-equiv/l., but the mean plasma K of 6·0 ± 0·6 m-equiv/l. did not differ significantly from the controls. The mean inulin space in cardiac muscle was 114 ± 9 ml./kg wet wt. and in skeletal muscle 53 ± 2 ml./kg wet wt., values not significantly different from the controls. The mean cell water in cardiac muscle was 657 ml./kg wet wt. and in skeletal muscle 720 ml./kg wet wt., representing only small increases in cell water compared with the control values of 646 and 717 ml./kg wet wt. Muscle Na is shown in Table 4 and K in Table 5. The mean inulin space obtained from three experiments was used to calculate the intracellular Na and K content.

TABLE 4. Total Na, intracellular Na fraction and intracellular Na concentration in adrenalectomized rats subjected to water and salt loading compared with adrenalectomized controls

	Cardiac muscle			Skeletal muscle		
	Total Na (m-equiv/kg wet wt.)	Intracellular Na (m-equiv/kg wet wt.)	Intracellular Na concn. (m-equiv/l.)	Total Na (m-equiv/kg wet wt.)	Intracellular Na (m-equiv/kg wet wt.)	Intracellular Na concn. (m-equiv/l.)
Water-loaded	30·5 ± 1·6	20·4	31·0	17·0 ± 0·9	11·9	16·8
Controls	33·6 ± 2·8	18·5 ± 1·8	28·8 ± 2·4	15·7 ± 0·8	10·6 ± 0·7	14·7 ± 1·1
Salt-loaded	39·7 ± 1·3	19·2 ± 1·9	30·8 ± 3·0	21·6 ± 1·5	14·5 ± 1·7	20·3 ± 2·0

TABLE 5. Total K content and intracellular K concentration in adrenalectomized rats with water and salt loading compared with adrenalectomized controls

	Cardiac muscle		Skeletal muscle	
	Total K (m-equiv/kg wet wt.)	Intracellular K concn. (m-equiv/l.)	Total K (m-equiv/kg wet wt.)	Intracellular K concn. (m-equiv/l.)
Water-loaded	96 ± 1·5	144	103 ± 4·0	144
Controls	101 ± 1·1	154 ± 1·8	102 ± 1·6	142 ± 1·6
Salt-loaded	113 ± 1·0	180 ± 3·2	102 ± 1·9	146 ± 2·7

From Table 4 it can be seen that with cardiac muscle there was a small fall in total Na, but no reduction in the intracellular fraction or the intracellular concentration. With skeletal muscle the values for total Na, intracellular Na and intracellular concentration were not lower than the controls. This is in clear contrast to the significant reduction that occurred in the non-adrenalectomized group subjected to water loading (Fig. 1). From Table 5 it can be seen that there was a loss of total K from cardiac muscle which was probably significant ($P < 0{\cdot}02$). There was no similar loss from skeletal muscle.

The effect of salt loading. The results of five experiments given below are compared with those for six adrenalectomized controls in each case. The mean plasma Na in these experiments was 163 ± 4 and the mean plasma K 7·6 ± 0·3 m-equiv/l., both values being significantly higher than the controls ($P < 0{\cdot}001$). The mean inulin space for cardiac muscle was 128 ± 11 ml./kg wet wt. and for skeletal muscle 51 ± 5 ml./kg wet wt., values not significantly different from the controls. The mean cell water for cardiac muscle was 620 ml./kg wet wt. and for skeletal muscle 691 ml./kg wet wt. There was a large variation in the values obtained for cardiac muscle and the results are not significantly lower than the controls ($P < 0{\cdot}3$). The reduction in cell water of skeletal muscle was possibly significant ($P < 0{\cdot}05$).

The values for muscle Na are shown in Table 4. It can be seen that with cardiac muscle there was a small increase in total Na, and smaller but not significant increases in the intracellular fraction and intracellular concentration of Na. With skeletal muscle there were increases in total Na, intracellular fraction and sodium concentration which were probably significant ($P < 0.01$ for total Na and $P < 0.05$ for intracellular fraction and Na concentration).

The results for total K and K concentrations are shown in Table 5. With cardiac muscle there was an increase in both the total and the final K concentration, both increases being highly significant ($P < 0.001$). With skeletal muscle no such increases in K occurred.

DISCUSSION

The present results show important differences in cardiac and skeletal muscle with regard to the transfer of electrolytes when these tissues are exposed to varying concentrations of sodium. In the living rat the present evidence suggests the existence of a homoeostatic mechanism which preserves the intracellular sodium content of cardiac muscle but not of skeletal muscle. Thus the intracellular sodium content of cardiac muscle was always remarkably stable whereas the intracellular sodium content of skeletal muscle varied extensively with changes of plasma sodium.

In the experiments with water loading there was no significant reduction in plasma potassium despite the increase in plasma volume that must have occurred. It is clear that the potassium needed to maintain this plasma concentration must have been derived from some reservoir and it is suggested that part of the reservoir may have been the intracellular potassium in muscle. When plasma sodium was raised by salt loading, the plasma potassium also rose. There was evidence of potassium entry into cardiac muscle but not into skeletal muscle, the latter finding confirming the experiments of Eichelberger (1941) and Conway & Hingerty (1948). It would seem therefore that cardiac muscle is more effective than skeletal muscle as a reservoir in potassium.

Following adrenalectomy the intracellular sodium content of cardiac muscle was normal and still not affected by water or salt loading. In skeletal muscle, however, although levels of sodium were normal after adrenalectomy, lowering of plasma sodium did not produce the fall in intracellular sodium found in the non-adrenalectomized animal. This suggests the presence of some defect in sodium extrusion.

SUMMARY

1. The effect has been studied of alterations of plasma Na on the Na and K content of cardiac and skeletal muscle in conscious rats.

2. With lowering of plasma Na induced by water loading there was a significant loss of cell Na from skeletal muscle but not from cardiac muscle,

whereas K was lost freely from cardiac muscle and to a less extent from skeletal muscle.

3. Adrenalectomy prevented the loss of skeletal muscle Na at low plasma Na levels but did not affect the loss of K from cardiac muscle.

4. With high plasma Na levels produced by salt loading, Na entered freely into skeletal muscle but not into cardiac muscle, whereas K entered freely into cardiac but not into skeletal muscle. Adrenalectomy did not affect these exchanges.

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THE EFFECTS OF STIMULATION OF VISCERAL AFFERENT NERVE FIBRES ON SOMATIC REFLEXES

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It is known that afferent nervous impulses from the viscera can reflexly elicit contractions of skeletal muscles (Miller & Simpson, 1924) and lead to an alteration in the excitability of somatic reflex arcs (Dusser de Barenne & Ward, 1937; Schweitzer & Wright, 1937).

Mellanby & Pratt (1939, 1940), using either decerebrate cats, or cats anaesthetized with chloralose, found that isometric contraction of the bladder was usually accompanied by contraction of the diaphragm, muscles of the abdominal wall, shoulder girdle and pelvic girdle, but sometimes it did not produce muscular activity, but quiescence of any already existing activity in these muscles. Downman & McSwiney (1946) showed that pinching or gently squeezing the large or small intestine, head of the pancreas or uterine horns of the decerebrate cat with acute transection of the spinal cord at the lower cervical level produced movement of the hind limbs. Similar visceral stimulation was less effective in decapitate cats, and in decerebrate cats hind-limb movements were rarely seen. Downman & McSwiney (1946) remarked on the variability of the responses obtained by visceral stimulation and they pointed out that frequent stimulation of the viscera or of the splanchnic nerve is often accompanied by the gradual disappearance of viscero-motor reflexes.

Downman (1955) described polysynaptic reflexes recorded from intercostal, lumbar and tibial nerves which were elicited by stimulation of the splanchnic nerves of decerebrate and acute spinal cats. He also found that polysynaptic reflexes could be recorded from intercostal nerves when a neighbouring intercostal nerve was stimulated, and that this reflex could be conditioned by a preceding splanchnic stimulus. In the decerebrate cat Downman found that splanchnic stimulation facilitated an intercostal-intercostal reflex. In decerebrate cats with an acute spinal transection the splanchnic conditioning volley inhibited the intercostal-intercostal reflex.

The experiments described in this paper were designed to make a more precise analysis of the effects of stimulation of visceral afferent nerve fibres upon monosynaptic and polysynaptic hind-limb reflexes.

METHODS

The experiments were carried out on adult cats weighing 1·7–5·9 kg. The majority were anaesthetized with intravenous chloralose, and in some of these the spinal cord was later transected at the level of C2. In some cats the spinal transection was performed and the brain pithed under ether anaesthesia, which was then discontinued.

A monosynaptic reflex was obtained either by stimulating a muscle branch of the sciatic nerve, or a dorsal spinal root, and by recording either from a ventral root, a branch of the sciatic nerve to a synergic muscle, or from the same nerve to which the stimulus was applied according to the procedure described by Lloyd (1943). Polysynaptic reflexes were obtained by stimulating branches of the sciatic nerve or a dorsal spinal root and recording either from a branch of the sciatic nerve to a muscle or from a ventral root. The recordings were obtained from platinum electrodes under liquid paraffin maintained at a temperature of 36–39° C.

Monosynaptic and polysynaptic test responses were conditioned either by visceral distension or by single shocks to the left splanchnic nerve or sympathetic chain. The splanchnic nerve was exposed retroperitoneally and divided at the coeliac ganglion, the central end being ligated and cleaned for stimulation. The sympathetic chain was exposed retropleurally after resection of the heads of one or two ribs, and was stimulated, sometimes in continuity, sometimes at its cephalic cut end. The viscus most commonly distended was the urinary bladder, but the rectum and uterus were distended on occasions. In some female cats the intravesical pressures were recorded by catheterizing them with double-bore polythene tubing, one bore being used to fill the viscus with Locke's solution at 38° C, while the other bore was connected to a mercury manometer. In male cats it proved impossible to pass a catheter of sufficient bore to measure the changes in intravesical pressure accurately. Because of the trauma to the bladder itself and its effects on the general condition of cats already debilitated by a lengthy operative procedure, it was thought inadvisable to tie catheters into the bladder.

In one experiment a uterine horn was distended in a similar manner to the bladder and the intra-uterine pressure was recorded. The rectum was distended by inflating a finger-stall, inserted *per rectum*, with 50 ml. of air.

The nerves were stimulated with rectangular pulses of 0·05–1 msec duration with an amplitude of 0·1–20 V delivered through an R.F. isolation unit. Hind-limb nerves were never stimulated at rates greater than 12/min, and the sympathetic chain and splanchnic nerve were stimulated at rates of about 1 or 2/min. Recorded responses were passed through a cathode-follower input stage to a.c.-coupled amplifiers and then to a double-gun oscilloscope. Care was taken to keep the grid current, from each input side of the balanced cathode-followers, to less than 10^{-11} A. When necessary the cats were immobilized with intravenous tubocurarine (1 mg/kg) or decamethonium iodide (0·2 mg/kg).

RESULTS

The effects of stimulating the sympathetic chain or splanchnic nerve on mono- and polysynaptic reflexes

Response to the conditioning stimulus. It was nearly always possible to record a response from a ventral root or from a nerve to a muscle of the hind limb if a sufficiently strong stimulus was applied to the sympathetic chain or to the splanchnic nerve. The latency of this response was 15–30 msec. In order to simplify the measurement and interpretation of the results of the experiments, the conditioning stimulus strength used was usually lower than that required to produce a detectable response.

Monosynaptic reflexes. When testing monosynaptic responses elicited from branches of the sciatic nerve, the interval between the conditioning stimulus and the test stimulus was varied from 0 to 70 msec. It is not always possible to be certain if a response is truly monosynaptic, and in the present experiments the responses were assumed to be monosynaptic if a sharp spike of short duration was obtained, and if, after allowing for the conduction time from the stimulating to the recording electrodes at a conduction velocity of 80 m/sec or more, the delay was 1 msec or less. For example, a response was assumed to be monosynaptic if, when stimulating and recording in the popliteal fossa, the total conduction time was less than 5 msec.

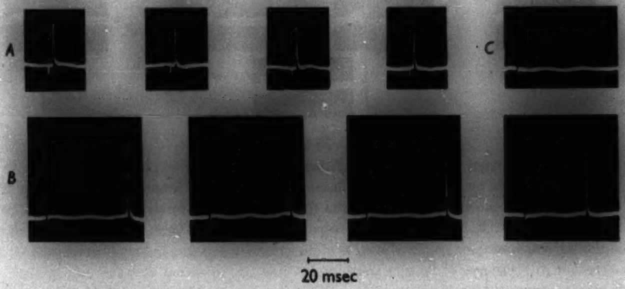

Fig. 1. Cat anaesthetized with chloralose. Effects of a preceding conditioning stimulus to the sympathetic chain on monosynaptic reflexes recorded from the L 6 ventral root and evoked by stimulation of the L 6 dorsal root. *A*, test responses; *B*, facilitation of test responses by the conditioning stimulus; *C*, conditioning response (none).

The effect on the monosynaptic test response of a conditioning stimulus to either the sympathetic chain or the splanchnic nerve was almost invariably a facilitation of the test response, although in some experiments the conditioning stimulus had no effect on the test response. This facilitation by a conditioning stimulus to the sympathetic chain is illustrated in Figs. 1 and 2. In Fig. 1 the response is recorded from the sixth lumbar ventral root following stimulation of the sixth lumbar dorsal root. The figure shows four control test responses (*A*), and the test responses during facilitation (*B*). The conditioning stimulus alone gave no response (*C*). In Fig. 3 the amplitude of the monosynaptic responses in this experiment are plotted against the time intervals between the conditioning and test stimuli. It will be seen that the facilitation starts at a stimulus interval of 25 msec, is maximal at 35 msec, and is no longer present at intervals greater than 60 msec.

In the experiment illustrated in Fig. 2 the response was recorded from a branch of the posterior tibial nerve to the small muscles of the foot, following stimulation of the same nerve. The figure shows four control test responses (*A*) and test responses during facilitation by conditioning stimulation of the sympathetic chain (*B*), which itself evoked a small response (*C*). The facilitation curve for this experiment is shown in Fig. 4*A*. It will be seen that the facilitation of the test responses starts when the stimulus interval is 12 msec,

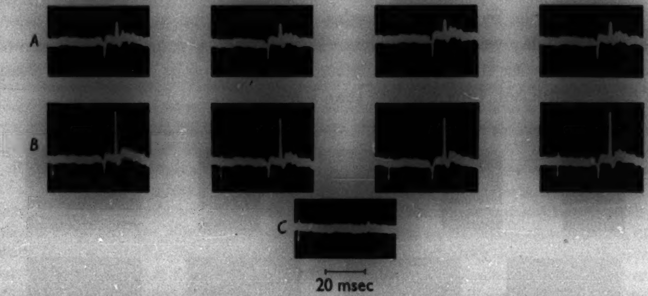

Fig. 2. Cat anaesthetized with chloralose. Effects of a preceding conditioning stimulus to the sympathetic chain on monosynaptic reflexes recorded from a branch of the posterior tibial nerve supplying the small muscles of the foot, evoked by stimulation of the same nerve. *A*, test responses; *B*, facilitation of test responses by the conditioning stimulus; *C*, small conditioning response.

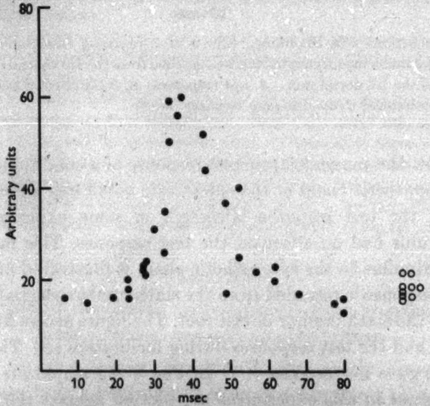

Fig. 3. Graph of the amplitudes, in arbitrary units, of the monosynaptic responses illustrated in Fig. 1 (ordinate) plotted against the interval between the dorsal root and sympathetic chain stimuli (abscissa). ○, test responses; ●, responses to interaction between conditioning and test stimuli.

becomes maximal at 25 msec and has almost disappeared at 60 msec. In Fig. 4 the facilitation curve obtained when the conditioning stimulus was applied to the splanchnic nerve (Fig. 4 *B*) is compared with that obtained when the conditioning stimulus was applied to the sympathetic chain of the same cat. Although a slight change in the excitability of the preparation caused the amplitude of the test responses to be slightly greater in *B* than in *A*, both curves show facilitation commencing when the interval between the conditioning and test stimuli is approximately 12 msec, and maximal at intervals between 20 and 40 msec.

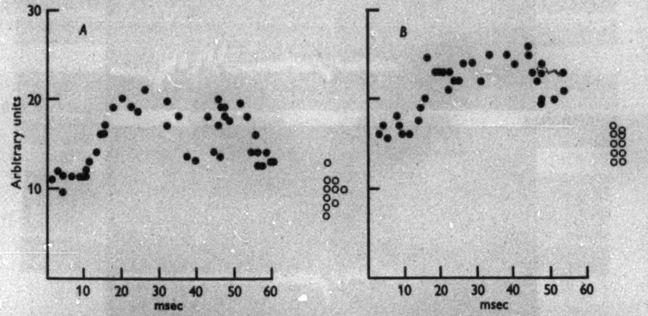

Fig. 4. Graphs of the amplitudes in arbitrary units of monosynaptic responses (ordinates) plotted against the interval between test and conditioning stimuli (abscissae). *A*, monosynaptic responses illustrated in Fig. 2, when conditioned by a stimulus to the sympathetic chain; *B*, monosynaptic responses evoked and recorded under identical conditions on the same cat as *A*, when conditioned by a stimulus to the splanchnic nerve. ○, test responses; ●, responses to interaction between conditioning and test stimuli.

Polysynaptic reflexes. When a conditioning stimulus was applied to the sympathetic chain or splanchnic nerve 10–70 msec before the test stimulus, polysynaptic reflexes elicited by stimulation of the dorsal spinal roots or hind-limb nerves were partially or totally inhibited. Facilitation of a polysynaptic reflex by a conditioning stimulus of the sympathetic chain or splanchnic nerve was never seen in either anaesthetized or spinal cats.

Fig. 5 illustrates the total inhibition of a polysynaptic reflex when the interval between conditioning and test stimuli was optimal for facilitation of the monosynaptic reflex evoked by the same test stimulus. The upper tracing (*A*) shows the monosynaptic (*m*) and polysynaptic (*p*) test responses recorded from the nerve to the semimembranosus muscle following stimulation of the posterior tibial nerve. The lower tracing (*B*) shows facilitation of the monosynaptic response and the total inhibition of the polysynaptic response when the splanchnic nerve was stimulated 37 msec previously. The response evoked in the nerve to the semimembranosus muscle by splanchnic stimulation is shown at *sp*.

Inhibition of the polysynaptic reflexes occurred when the conditioning and test stimuli were separated by intervals which were too long to affect the monosynaptic reflexes elicited by the same test stimulus.

No qualitative difference in the effects of conditioning stimulation of the sympathetic chain or splanchnic nerve on hind-limb reflexes was seen between cats anaesthetized with chloralose and cats which had a high spinal section followed by pithing of the brain.

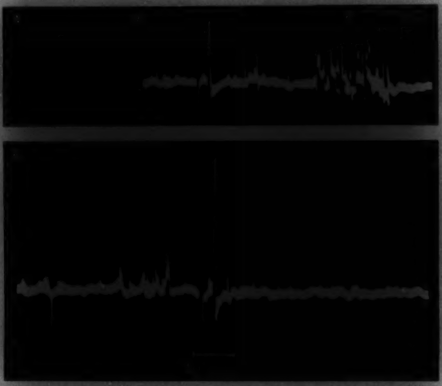

Fig. 5. Cat anaesthetized with chloralose. Effects of a preceding conditioning stimulus to the sympathetic chain on monosynaptic (*m*) and polysynaptic (*p*) responses recorded from the nerve to the semimembranosus muscle and evoked by stimulation of the posterior tibial nerve: the direct response from stimulation of the sympathetic chain is shown at *sp*. *A*, test responses; *B*, facilitation of the monosynaptic and inhibition of the polysynaptic responses by the conditioning stimulus.

The effects of visceral distension of mono- and polysynaptic reflexes

Distension of the viscera did not produce any detectable response in ventral roots or hind-limb nerves. The effects of visceral distension on mono- and polysynaptic reflexes elicited from the hind limb were more variable than the effects of sympathetic and splanchnic stimulation on these reflexes. In some experiments visceral distension produced no effects, in others the effects were doubtful, and in others definite effects were obtained. Not only was there variability between preparations but it was sometimes impossible to reproduce the effects of visceral distension more than once or twice in the same cat.

The volumes of Locke's solution necessary to produce effects on somatic reflexes by distension of the bladder varied from 20 to 100 ml. In those experiments in which it was possible to record the pressure inside the bladder, pressures of 30–100 mm Hg produced effects on the hind-limb reflexes. The actual pressure at which effects on the hind-limb reflexes were obtained varied from animal to animal and no definite conclusions could be drawn about the threshold pressure which produced these effects. Distension of the uterine horn with 10 ml. of Locke's solution at a pressure of 80–120 mm Hg produced effects on the hind-limb reflexes. The pressure at which rectal distension produced similar effects was not recorded. The effects of visceral distension on hind-limb reflexes were similar in anaesthetized and spinal cats.

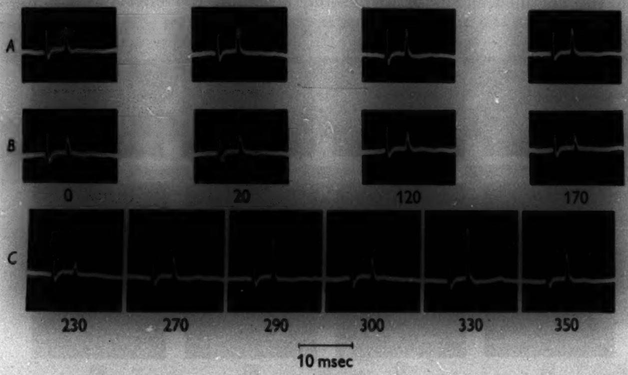

Fig. 6. Cat anaesthetized with chloralose. Effects of distension of the bladder on monosynaptic responses recorded from a branch of the posterior tibial nerve supplying the small muscles of the foot and evoked by stimulation of the same nerve. *A*, test responses; *B*, inhibition of test responses during distension of the bladder with 80 ml. Locke's solution; *C*, recovery of test responses after emptying the bladder, with intermittent facilitation. The numbers below the responses show the time in seconds after the commencement of bladder distension, which was maintained for 170 sec.

Monosynaptic reflexes. In the majority of experiments bladder distension had no significant effect on the amplitude of the monosynaptic reflexes. In some experiments bladder distension reduced the amplitude of the monosynaptic spike by about 50%. This inhibition of the monosynaptic response occurred almost immediately the bladder was distended, and when the latter was emptied the reflex did not regain its former height immediately, but returned slowly to normal over a period of about 1 min. In the experiment illustrated in Fig. 6 monosynaptic responses were recorded from a branch of the posterior tibial nerve to the small muscles of the foot, following stimulation of the same nerve. In this figure, four control test responses are shown (*A*) and four test responses during distension of the bladder for a period of 170 sec (*B*). It will be seen that, in this experiment, distension of the bladder reduced the amplitude of the monosynaptic response by approximately 50%.

When the bladder was emptied the reduction in the monosynaptic response persisted for about 60 sec, as shown in the first frame of (*C*). This was followed by a period of about 150 sec during which the monosynaptic response was frequently increased (*C*).

In this experiment, as in those illustrated by Figs. 7 and 8, it was not possible to know the exact time when the visceral distension became effective, as the test response was evoked at intervals not less than 5 sec and an even longer time was taken in distending the viscus. Zero time in Figs. 6–8 is taken from the commencement of distension, although the effect would not be immediate.

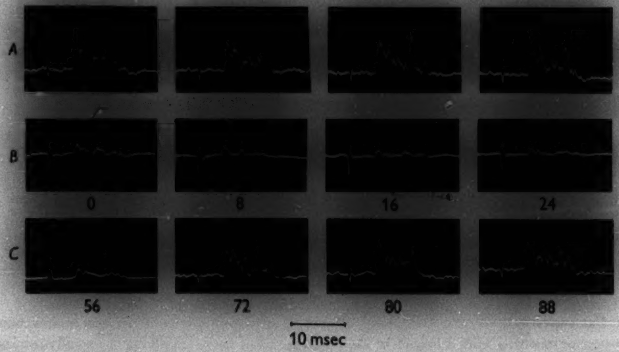

Fig. 7. Cat anaesthetized with ether and spinal cord transected at C2. Effects of distension of the rectum on monosynaptic and polysynaptic reflexes recorded from the nerve to the biceps femoris muscle and evoked by stimulation of the posterior tibial nerve. *A*, test responses; the initial spike in each record is the monosynaptic response; *B*, inhibition of test responses during distension of the rectum; *C*, recovery of test responses after cessation of rectal distension. The numbers below the responses show the time in seconds after the commencement of rectal distension, which was maintained for 24 sec.

The effects of distension of the rectum on the monosynaptic responses were similar to those of bladder distension. In Fig. 7, monosynaptic and polysynaptic responses were recorded from the nerve to biceps femoris following stimulation of the posterior tibial nerve. The monosynaptic response is represented by the initial spike in each record. It will be seen that the initial spike is depressed by distension of the rectum (*B*).

Polysynaptic reflexes. In about half the experiments distension of the bladder or rectum had no detectable effect on polysynaptic reflexes elicited from the hind limb. In the remainder the polysynaptic reflexes were partially or totally inhibited. This inhibition occurred almost immediately the viscus was distended and the later components of the polysynaptic responses were usually inhibited more readily and completely than the early components. The time taken for the polysynaptic reflex to reach its original level after the viscus was emptied varied from a few seconds in some experiments to about 5 min in others. In approximately one third of the experiments there was a partial recovery of the polysynaptic reflex when the bladder or rectum was still distended. The effects of distension of the bladder on polysynaptic reflexes is illustrated in Fig. 8. In this figure three control test responses recorded from the nerve to semimembranosus following stimulation of the posterior tibial nerve are shown (*A*). The bladder was distended for 120 sec, during which time the test responses were inhibited (*B*), recovering slowly when the bladder was emptied (*C*).

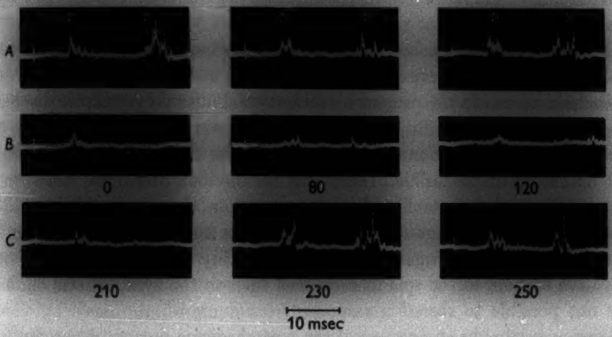

Fig. 8. Cat anaesthetized with chloralose. Effects of distension of the bladder on polysynaptic reflexes (*p*) recorded from the nerve to the semimembranosus muscle evoked by stimulation of the posterior tibial nerve. *A*, test responses; *B*, inhibition of test responses during distension of the bladder with 80 ml. Locke's solution; *C*, recovery of test responses after emptying the bladder. The numbers below the responses show the time in seconds after the commencement of bladder distension, which was maintained for 120 sec.

The effects of distension of the rectum on polysynaptic reflexes is shown in Fig. 7, in which the later components of the response are inhibited during and after the period of rectal distension (*B*).

In one experiment in which the uterus was distended, polysynaptic reflexes only were recorded from the nerve to semitendinosus following stimulation of the posterior tibial nerve, and these reflexes were inhibited by the uterine distension.

DISCUSSION

One of the difficulties in obtaining clear-cut results in this investigation is due to the lengthy and traumatic operative procedure which is necessary to perform the experiments. In addition, it is inadvisable to stimulate the sympathetic chain and splanchnic nerve more than 1 or 2 times/min. The traumatic effects of the experiments, together with the deleterious effects of hyperventilation on viscero-motor reflexes previously noted by Downman & McSwiney (1946), may account in part for the fact that visceral afferent activity sometimes failed to affect somatic reflexes. However, these factors cannot account for the variability in the results.

As suggested by other authors (Schweitzer & Wright, 1937; Downman, 1955) it is extremely unlikely that the effects of stimulation of visceral afferent nerve fibres on hind-limb reflexes are merely secondary to the changes in respiration or blood pressure which such stimulation produces. The somatic motor effects are produced before any changes in respiration or blood pressure are manifest. Moreover, visceral afferent stimulation has different effects on mono- and polysynaptic hind-limb reflexes, which cannot be explained by changes in respiration or blood pressure.

Downman (1955) has shown that a reflex discharge of long latency can be evoked in the 'tibial nerve at the knee' of the spinal cat by stimulation of the splanchnic nerve. In the present investigation similar polysynaptic discharges produced by stimulation of the sympathetic chain or splanchnic nerve were recorded from nerves to muscles acting as flexors of the hip, knee and ankle, and extensors of the ankle. There was no obvious difference between the responses recorded from hind-limb nerves when the sympathetic chain or splanchnic nerve was stimulated although it was easier to obtain results from stimulation of the sympathetic chain, probably because there was more operative trauma to the splanchnic nerve than to the sympathetic chain. It was not possible to record a response from a hind-limb nerve by visceral distension, probably on account of the asynchronous nature of the discharge produced by the latter. In all the successful experiments in this investigation there was no difference in the effects of visceral afferent stimulation on hind-limb reflexes between anaesthetized and spinal cats.

The facilitation of monosynaptic reflexes produced by electrical stimulation of the sympathetic chain or splanchnic nerve indicates that stimulation of these visceral afferents produces increased excitatory activity in descending spinal pathways, which must be presumed to consist of many interneurones because of the long latency of the sympathetic—or splanchnic—evoked discharge. Stimulation of visceral afferent fibres by visceral distension does not have any such facilitatory activity; it either has no effect on the monosynaptic response or inhibits it. These different results of the two types of conditioning stimulation cannot be explained by differences in conduction velocity in the sympathetic chain and splanchnic nerve, on the one hand, and the afferent nerves from the pelvic viscera, on the other. Nor will differences in peripheral conduction velocity explain the fact that sympathetic and splanchnic stimulation facilitates the monosynaptic, but inhibits the simultaneously evoked polysynaptic reflex, whereas visceral distension inhibits both reflexes. Facilitation of the monosynaptic reflex with inhibition of the simultaneously evoked polysynaptic reflex produced by conditioning stimulation of the sympathetic chain or splanchnic nerve cannot be explained by supposing that the conditioning stimulus causes more motoneurones to discharge, producing a 'maximal' monosynaptic response and leaving the motoneurones refractory to the late-arriving polysynaptic volley. The polysynaptic response is inhibited at stimulus intervals when the monosynaptic response is unchanged.

Electrical stimulation of the sympathetic chain or splanchnic nerve produces a relatively synchronous volley, whereas visceral distension produces a long-lasting, asynchronous discharge. The duration of the inhibition of the polysynaptic reflex produced by conditioning stimulation of the sympathetic chain or splanchnic nerve is appreciably longer than the duration of the facilitation of the monosynaptic reflex produced by the same stimulation. If one assumes that the durations of the central inhibition and facilitation produced by visceral distension are similar, then it is possible to explain the differences between the effects of stimulation of the sympathetic chain or splanchnic nerve, and the effects of visceral distension by the differences in the temporal pattern of the afferent volleys. It may well be that if any facilitatory effects are produced by visceral distension they are unable to overcome the continuing and long-lasting inhibition produced by the asynchronous discharge from pelvic visceral afferents, i.e. the central inhibitory state produced by each succeeding impulse from pelvic visceral afferents depresses the excitability of the reflex arc for such a duration that it is unable to respond to a briefer facilitation which may be produced at the same time.

A phenomenon which merits further investigation is the long-lasting inhibition of spinal reflexes which is produced by bladder distension: this inhibition may last for several minutes after the distension has ceased. It may be that bladder receptors continue to discharge when the distension ends, although there is no reported evidence of such a continuing discharge. If a continuing discharge from bladder receptors does not occur, one is led to conclude that bladder distension produces a long-lasting excitatory state probably in inhibitory interneurones. In this connexion, it is interesting to note that Freeman (cited by Fulton, 1950) states that if the bladder of spinal paraplegic patients is allowed to distend even once only, a prolonged inhibition of spinal reflexes results, lengthening the period of spinal shock.

SUMMARY

1. Experiments on cats are described in which test stimuli were applied to the nerves of the hind limb and monosynaptic and polysynaptic reflexes were recorded from ventral spinal roots or peripheral somatic nerves.

2. These monosynaptic and polysynaptic reflexes were conditioned by electrical stimulation of the sympathetic chain or splanchnic nerve, and by distension of the bladder, rectum or uterus.

3. Electrical stimulation of the sympathetic chain or splanchnic nerves facilitated the monosynaptic reflexes and inhibited the polysynaptic reflexes elicited from the hind limb.

4. Visceral distension had either no effect on the monosynaptic reflexes or it inhibited them. Visceral distension inhibited the polysynaptic reflexes. This inhibition sometimes lasted for several minutes after the distension ceased.

5. The difference in effects on hind-limb reflexes between electrical stimulation of visceral afferent nerves and visceral distension could be explained by the difference in the temporal pattern of the afferent discharges evoked by these two types of stimulation.

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THE SITE OF ACTION OF PROCAINE ON THE ISOLATED SPINAL CORD OF THE FROG

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It is frequently reported that, in man, the onset of spinal anaesthesia by the intrathecal injection of such local anaesthetics as procaine or allied compounds produces a selective blocking of different types of sensation and reveals a difference in the susceptibility of motor and sensory functions.

This selective action of spinal anaesthetics could be due to differences in the susceptibility of either the axons in the dorsal and ventral roots, or the intramedullary branches of these axons and their associated neurones. The experiments described in this paper were designed to determine whether there was a preferred site of action for procaine on intramedullary structures. For such an investigation, the preparation of the cat's spinal cord *in situ* has the disadvantage that, with it, it is difficult to ensure that the local anaesthetic solution completely surrounds the cord, and that it is not being constantly diluted by the cerebrospinal fluid. The isolated frog's spinal cord provides a simpler and more satisfactory preparation, as it can be completely immersed in the test solution, while the dorsal and ventral roots are kept clear for stimulating and recording.

METHODS

The spinal cord of a frog, below the first cervical segment, was removed complete with the dorsal and ventral roots of the 9th and 10th segments, and placed in a Perspex bath (Fig. 1) through which circulated Ringer's solution equilibrated with 95% O_2 + 5% CO_2. The composition of the Ringer's solution was: NaCl, 0·7; KCl, 0·014; $CaCl_2$, 0·012; $NaHCO_3$, 0·02 g/100 ml. H_2O. The dorsal and ventral roots were separated and lifted on to platinum electrodes in the covering layer of paraffin for stimulating and recording. The Ringer's solution was earthed close to the spinal cord. Procaine HCl was added to the Ringer's solution to the concentration required and the effects recorded 20–30 min later. The temperature of the bath was maintained at from 17 to 20° C. Stimuli were obtained from a Grass square-wave stimulator at a frequency of approximately once every 10 sec. Records were made on 35 mm film from a Cossor oscilloscope after d.c. amplification.

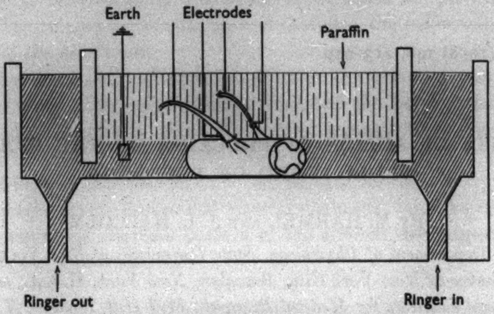

Fig. 1. Cross-sectional view of Perspex bath used to maintain isolated frog spinal cord. Rate of flow of Ringer's solution, 100 ml./hr.

RESULTS

Fig. 2 illustrates the results obtained in a typical experiment. The records in column *A* are the responses of the 10th ventral root to a stimulus applied to the 10th dorsal root on the same side. The only consistent effect recorded with concentrations of 1×10^{-4} and 3×10^{-4}, as used to obtain the second and third records, is a slight delay in the onset of the polysynaptic portion of the response, indicated by the changes in the inflexion on the rising phase of the ventral root response. This inflexion is marked by an arrow in column *A*. The slight reduction in height in the third record is within the limits of experimental error. Procaine in a concentration of 1×10^{-3} (bottom record) is required in order to reduce the height of the response to a height significantly less than the height of the control (top record). The responses recorded from the 9th dorsal root after the same stimulus to the 10th dorsal root (column *B*) are more susceptible to procaine. A concentration of 1×10^{-4} procaine, which had no effect on the height of the ventral root response, definitely diminished the dorsal root response (2nd record), and there is further diminution by higher concentrations (3rd and 4th records, column *B*). The most susceptible root response, however, is that of the dorsal root to an antidromic volley in the ventral root of the same side and segment (column *C*). Procaine at a concentration of 1×10^{-4} markedly diminishes the response (2nd record) and at a concentration of 3×10^{-4} abolishes it (3rd record).

Because this response to an antidromic ventral root volley seemed the most susceptible to procaine, the spread of this volley over the ventral horn cells and their dendrites was further examined by a method of surface recording similar to that used by Lloyd (1951) in the spinal cord of the cat. Fig. 3 shows the records from six points on the surface of the cord between the dorsal root entry and the ventral root exit, before (column *A*), and after (column *B*), bathing the cord in procaine at a concentration of 3×10^{-4}.

The pattern of the surface responses is interpreted as follows. The first wave (after the stimulus artifact) marked a is always positive at all points on the surface of the cord, and negative only when the recording electrode is placed on the ventral root. Hence it signals the presence of the incoming volley in the ventral root axons. This a-wave is followed by a short wave marked s that is negative at points on the ventral region of the lateral surface of the cord (points 4–6, Fig. 3) and positive at points on the dorsal region (points 1 and 2, Fig. 3). Such a distribution of positive and negative waves strongly suggests that it signals the invasion of the soma of the ventral horn cells.

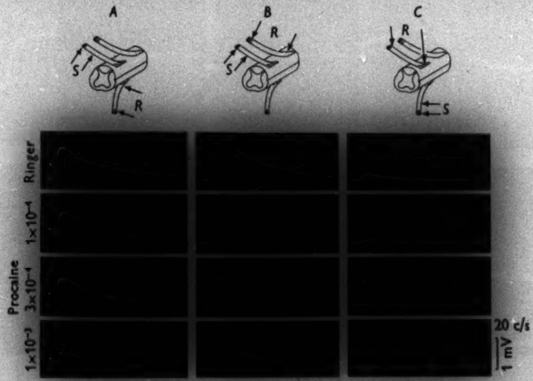

Fig. 2. Records of root potentials of an isolated frog spinal cord. Diagrams at the top of each column indicate the position of stimulating electrodes (*S*) and recording electrodes (*R*). Column *A*, records obtained from the 10th ventral root by stimulating the 10th dorsal root. Column *B*, records obtained from the 9th dorsal root by stimulating the 10th dorsal root. Column *C*, records obtained from the 10th dorsal root by stimulating the 10th ventral root. First record in each column obtained with Ringer's solution only bathing the special cord. Second record in each column, after bathing for 30 min in procaine 1×10^{-4}. Third record, after 30 min in procaine 3×10^{-4}. Lowest record, after 30 min in procaine 1×10^{-3}. Arrows on records in column *A* indicate points of commencement of the polysynaptic component of the ventral root response.

The third wave, marked d, is always negative and its characteristics suggest that it signals the invasion of dendrites. Its appearance is increasingly delayed as the recording point is moved away from the region of the ventral horn cells. (Compare d in the records from points 1 and 6, Fig. 3, column *A*.) At the more distant points (e.g., point 1, Fig. 3), this negative d-wave is preceded by a positive wave that is coincident with the negative d-wave recorded at points adjacent to the ventral horn. The more distant branches of the dendritic tree evidently act as 'sources' of current to the 'sinks' in the proximal branches, before they in turn are invaded, become 'sinks' and give rise to a delayed negative deflexion. The presence of a negative d-wave in all the records in column *A*, Fig. 3, indicates that some dendrites close to these points on the surface were invaded by the antidromic volley.

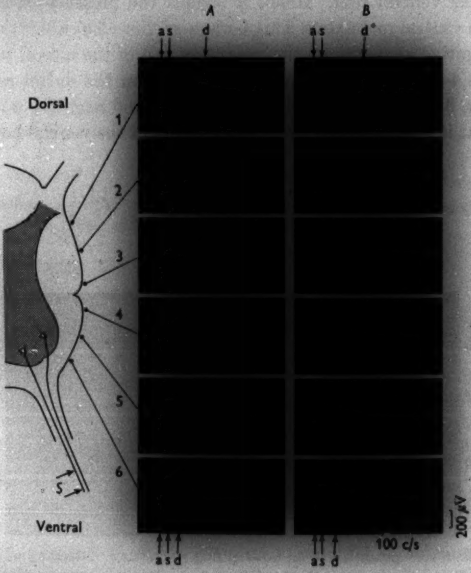

Fig. 3. Records obtained from points at mid-segmental level on the surface of the cord as indicated on the diagrammatic section on the left. The cord and a proximal part of the ventral root were fully immersed in the Ringer's solution. Stimulus applied, at *S*, to the ventral root to initiate an antidromic volley. Column *A* before, column *B* after, 30 min in procaine 3×10^{-4}. Stimulus artifact indicated by dot and line. a-, s-, and d-waves, see text.

The records in column *B*, Fig. 3, were taken 20 min after procaine 3×10^{-4} had been added to the perfusing fluid. In this experiment, unlike that illustrated in Fig. 2, such a concentration abolished the dorsal root response to an antidromic volley, but had no effect on either the dorsal or ventral root responses to an orthodromic dorsal root volley. The records from the surface of the cord, however, show that the d-wave is markedly decreased while the a- and s-waves are not significantly altered. The diminution of the d-wave in the regions proximal to the cell bodies (records 5 and 6, Fig. 3) coincides with a decrease in the positive wave in the records from the more distant regions (records 1 and 2, Fig. 3). The positive wave recorded at these more distant points is followed by only a minute negative wave. This shows that, as a result of the action of procaine, the more distant branches of the dendritic tree can still act as a 'source' to the smaller 'sink' in the proximal branches, while very few of the more distant branches are themselves invaded.

Procaine interferes with the normal facilitatory action of a dorsal root volley on an antidromic ventral root volley. This facilitation is confined to the dendritic component as illustrated by Fig. 4. Record *A*, in Fig. 4, is the normal response to an antidromic volley recorded at a point corresponding to position 4 in Fig. 3, while *B* is the response to the same volley at the same point 35 msec after a maximal dorsal root volley. The a- and s-waves are unchanged, but the d-wave is greatly increased, i.e. the antidromic volley has now spread into many more branches of the dendritic tree. After bathing the cord in procaine 1×10^{-4} for 30 min the antidromic response alone is slightly depressed (Fig. 3 *C*) and the extent to which the d-wave can be facilitated greatly diminished (Fig. 3 *D*). At this concentration of procaine, the conditioning facilitating volley was unaffected.

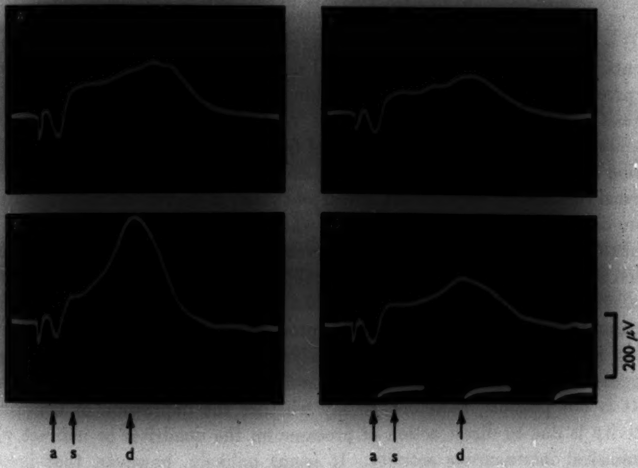

Fig. 4. The response of a point in the middle of the lateral surface of a frog's spinal cord to an antidromic volley in the ventral root, Records *A* and *B* before, *C* and *D* after, bathing the cord in procaine 1×10^{-4} for 30 min. Records *A* and *C* without, *B* and *D* with, a facilitating dorsal root volley initiated 35 msec earlier. a, s and d, as in Fig. 3 and in text.

DISCUSSION

The effects of low concentrations of procaine are primarily on the dendrites of the ventral horn cell. This is clearly indicated by the block of an antidromic volley before it spreads very far beyond the soma, and the inability of an orthodromic volley, apparently unaffected, to facilitate this spread. More weight is given to this hypothesis by the finding that a dorsal root response to a neighbouring dorsal root volley is little affected by concentrations of procaine that block the dorsal root response to an antidromic ventral root volley. Presumably the terminal ramifications of the afferent fibres are less susceptible than the dendrites of the ventral horn cells.

It is not possible to assess the role played by interneurones in these experiments. If they provide the preferential site of action for low concentrations of procaine one would expect that blocking their activity would diminish the spread of the antidromic volley in the ventral root only if they normally maintained a continuous background of facilitating bombardment. The cessation of such a background of facilitation would be at once obvious on the normal ventral root response to a dorsal root volley if the ventral root discharge were dependent on the integrity of the whole motoneurone. This, however, is probably not the case as a nearly normal ventral root discharge can be obtained when the dendrites cannot be antidromically activated. A large factor of safety is seemingly maintained so that activation by the afferent volley of soma and proximal dendrites alone is sufficient to produce a normal response. The slight delay in the initiation of the later, polysynaptic, portion of the response suggests that the presynaptic volleys are not quite so adequate as normally.

It is not possible, therefore, to say whether the depression of dendritic activity is due to direct action of procaine on the dendrites or indirect via diminished interneurone activity. It seems unlikely, however, that interneurones would show much continuous activity in such an isolated preparation as used here, especially as the tension of CO_2 provided from the perfusing fluid is higher than that normally found in the frog's spinal cord and would be expected therefore to depress the over-all activity of interneurones. The results of these experiments therefore strongly suggest that the dendrites of the motoneurones are the most susceptible to the action of procaine.

The reason for this susceptibility does not necessarily lie in a specific chemical difference between dendrites and other structures. Two other possible explanations should be borne in mind. One is that the differences in susceptibility depend upon variations in the safety factor for conduction of nerve volleys in different units. The excitation process, normally, may be only just adequate for conduction over dendrites, whereas for firing a motoneurone it may be many times that needed to reach threshold. In these circumstances, the procaine would be more effective on dendritic conduction though equally distributed over all the elements in the cord.

The other possible explanation is that the procaine diffuses unequally throughout the cord and hence units that ramify over the widest field are the ones most likely to be affected by the procaine. This would account for the greater susceptibility of the motoneurone dendrites which ramify extensively through several segments of the cord.

SUMMARY

1. The sensitivity to procaine of different parts of the isolated frog's spinal cord was observed by recording the electrical responses of dorsal and ventral roots and the spinal cord to orthodromic and antidromic nerve volleys.

2. The most sensitive responses were:

(*a*) the dendritic response of the motoneurone to an antidromic, ventral root volley;

(*b*) the dendritic response of the motoneurone to an antidromic volley after facilitation by an orthodromic, dorsal root volley;

(*c*) the dorsal root response to an antidromic, ventral root volley.

3. The most sensitive responses depend primarily on the integrity of the motoneurone dendritic tree, though the possibility of the failure of interneurones, if active, playing some part in the recorded changes cannot be excluded.

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PHOTOSENSITIVE PIGMENTS FROM THE RETINAE OF CERTAIN DEEP-SEA FISHES

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The bizarre morphology of deep-sea fishes has fascinated students and led to the inference of functional as well as structural modification. Although the strange eyes of deep-sea fishes have been the subject of histological studies (e.g. Brauer, 1908) and of scientific speculation for many years, experimental work was not attempted until very recently. Then light-sensitive retinal pigments of several species of deep-sea fishes were examined by measuring the gross changes in optical density following exposure of the retinae to light (Denton & Warren, 1956). The spectral location of these pigments was reported to be about 20 mμ less than that of rhodopsin, thus placing the peak absorptions at about 480 mμ. The name chrysopsin was proposed for these pigments.

In recent years the analysis of retinal photosensitive pigments has been greatly advanced by the method of partial bleaching, which makes it possible to test the homogeneity of the photolabile component present in retinal extracts (Dartnall, 1952). The present investigation was undertaken to explore the retinal pigments of a number of deep-sea fishes using these modern methods. A preliminary note of this study has already appeared (Munz, 1957).

METHODS AND APPARATUS

Collection and identification of material

Through the kindness of Dr C. L. Hubbs, of the Scripps Institution of Oceanography, the eyes of bathypelagic fishes collected on an expedition of the Scripps vessel *Paolina-T*, in which the author took part, were made available for this study. Organisms were collected in a 10 ft. Isaacs-Kidd mid-water trawl on the nights of 9 and 10 February 1957, at depths from 280 to 380 fathoms near Guadalupe Island, Baja California, Mexico. The net was brought up on deck by moonlight, and its luminescent blue-glowing contents were dumped into a bucket, which was carried into the ship's laboratory. Here by dim red light Dr Hubbs sorted the collections and made field identifications of the fishes. The author then removed the eyes and, where possible, the retinae by red light, placed them in distilled water or 4% potassium alum solution and kept them frozen in lightproof containers until returning to the laboratory (8–9 days). The fishes were preserved after removal of their eyes and were identified by Mr A. W. Ebeling of the Scripps Institution of Oceanography. Table 1 lists the species examined in this study and certain details of preparation of the retinal extracts.

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* Predoctoral fellow, National Institute of Neurological Diseases and Blindness, U.S. Public Health Service. This work is a portion of a dissertation for the Ph.D. degree at the University of California, Los Angeles.

TABLE 1. Spectral characteristics of retinal extracts

Species	Family	No. of eyes	Extract sample	Density (700 mμ)	Maximum density	$\lambda_{max.}$ (mμ)	Ratio	Adjusted $\lambda_{max.}$ (mμ)
Bathylagus wesethi	Argentinidae	8*	1A	0·004	0·460	483	0·39	484
			1B	0·004	1·060	485	0·37	486
			1C	0·028	0·672	482	0·56	485
Argyropelecus affinis	Sternoptychidae	2	2A†	−0·003	0·562	477	0·43	478
			2B	−0·001	0·372	477	0·43	478
			2C	0·002	0·143	476	0·54	478
Sternoptyx obscura	Sternoptychidae	6*	3A	0·006	0·552	484	0·48	485
			3B	0·001	0·594	484	0·50	486
			3C	0·001	0·148	484	0·59	487
Stomias atriventer	Stomiatidae	4	4A	0·020	0·202	475	0·95	—‡
Lampanyctus mexicanus	Myctophidae	16§	5A	0·047	1·056	486	0·65	490
			5B	0·093	0·553	468	0·98	—‡
Melamphaes bispinosus	Melamphaidae	16§	6A	0·050	1·121	485	0·63	488
			6B	0·035	0·552	484	0·68	488

* Retinal material frozen in 4% potassium alum solution; otherwise in distilled water.

† After the absorption spectrum was measured, this extract was divided and diluted into samples 2B and 2C.

‡ The high ratio prevented estimation of the true $\lambda_{max.}$.

§ Whole eyes extracted; otherwise retinae were removed before extraction.

Preparation of retinal extracts

Digitonin extracts of the whole retinal material were prepared with methods used previously (Munz, 1956). Where the eyes were small, the whole eyes were mashed, then washed and extracted in the same manner as the retinae. Retinae which had been frozen in distilled water were thawed and then hardened for a few minutes in 4% potassium alum solution (this step was omitted with the material which had been frozen in alum solution). They were then washed twice in distilled water, being centrifuged lightly each time to allow separation from the supernatant fluid. After a further wash in alkaline (pH 8·6) borate-KCl buffer, the retinal material was extracted with two successive portions of 2% aqueous digitonin solution made up in borate-KCl buffer (pH 8·3). The extracts were stored in darkness at 10° C.

Measurement of absorption spectra and bleaching apparatus

After centrifugation the retinal extracts were analysed in a Beckman DU spectrophotometer with photomultiplier attachment. Two per cent digitonin solution was used as a blank; the temperature was regulated at 20 ± 1° C. Optical density of the extracts (1 cm light path) was measured from 700 to 320 mμ in 20 mμ intervals, followed by an interlaced return series of measurements, also at 20 mμ intervals. A Bausch and Lomb grating monochromator with interference filters placed in the exit light path provided narrow-band coloured light (half band-widths between 10 and 15 mμ) which was used in bleaching the extracts. Following exposure to light the extracts were again placed in the spectrophotometer and the absorption spectrum measured as before. In this way the effects on each extract of several bleaching wave-lengths were studied successively. White light from a 60 W tungsten bulb was also employed as a bleaching source.

RESULTS

The photosensitive pigment of Argyropelecus

Two specimens of the hatchet-fish *Argyropelecus affinis* Garman were obtained, and a digitonin extract was prepared from the right retinae. The absorption maximum of this extract was 477 mμ (see Table 1 and curve 1 of Fig. 1*A*). The ratio of optical densities of the minimum (at 390 mμ) to maximum was 0·43. This $D_{\text{min.}}:D_{\text{max.}}$ ratio is related to the absorption maximum of visual

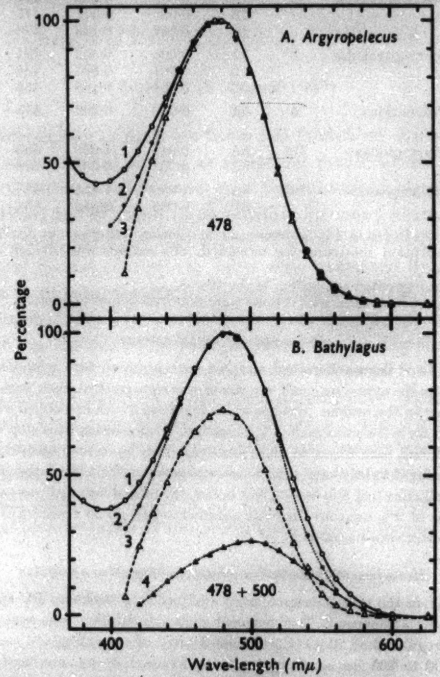

Fig. 1 *A*. Curve 1, absorption spectrum of extract sample 2 A of *Argyropelecus*; curve 2, constructed from Dartnall's nomogram, assuming a maximum at 478 mμ; curve 3, NH_2OH experiment, difference spectrum after exposure of sample 2 C to green light (560 mμ).

Fig. 1 *B*. Curve 1, absorption spectrum of extract sample 1 A of *Bathylagus*; curve 2, constructed from Dartnall's nomogram, assuming a maximum at 485 mμ; curves 3 and 4 from NH_2OH experiment with sample 1 C. Curve 4 is difference spectrum obtained by exposure to red light (631 mμ); curve 3 is difference spectrum after exposure to 560 mμ. Curves 3 and 4 are the same as curves 1–2 and 6–7 of Fig. 3 *B* but scaled so as to approximate in sum the total difference 1–7.

pigment extracts (Crescitelli & Dartnall, 1954, Fig. 8). From this curve true $\lambda_{max.}$ of the hatchet-fish pigment was estimated to be 478 mμ. Curve 2 of Fig. 1*A* is the theoretical curve constructed for a visual pigment maximal at 478 mμ from Dartnall's nomogram (1953). The nomogram is based on his observation that the difference spectra of all known visual pigments have the same shape when plotted on a frequency scale. The theoretical curve and absorption spectrum (curve 1) coincide except at wave-lengths less than 450 mμ, where curve 1 lies above the theoretical curve, presumably owing to the presence of blue-absorbing impurities. Hydroxylamine (NH_2OH) combines with retinene to form the oxime (Hubbard & Wald, 1952), which has a $\lambda_{max.}$ at a shorter wave-length than retinene, thus minimizing the effect on the positive portion of the difference spectrum of the product of bleaching (Crescitelli, 1956). The NH_2OH difference spectrum (curve 3) of the hatchet-fish is also in close agreement with curve 2 except in the short wave-length portion, where the NH_2OH difference spectrum falls more rapidly owing to the presence of the product. These three sources of information, i.e. $\lambda_{max.}$ of absorption spectrum, agreement with theoretical curve for a visual pigment maximal at 478 mμ, and $\lambda_{max.}$ of the NH_2OH difference spectrum, all combine to show that $\lambda_{max.}$ of the hatchet-fish retinal pigment is 478 ± 1 mμ.

Partial bleaching with lights of several wave-length compositions demonstrated the photosensitivity and homogeneity of the *Argyropelecus* extract (see Table 2). No evidence for a mixture of light-sensitive pigments was obtained; each stage of the bleaching indicated that the same component had been affected by exposure to light. The difference spectrum without NH_2OH was maximal at 481 or 482 mμ and at 478 mμ with NH_2OH. Fig. 2 shows the results of a partial bleaching experiment with extract 2C. The difference spectra (Fig. 2*B*) have the same $\lambda_{max.}$ except for the last, which showed a small shift toward shorter wave-lengths caused by exposure to isomerizing light. Attention has been called to this effect before (Crescitelli, 1956; Munz, 1956), and it is not believed to indicate the bleaching of any additional pigment of the same type as the one maximal at 478 mμ.

The nature of the carotenoid chromophore of a visual pigment molecule is suggested by the spectral position of the product of bleaching, and this relationship has been used by Crescitelli (1956) to characterize the carotenoid systems of amphibian and gecko visual pigments. The product of bleaching of the *Argyropelecus* pigment absorbed light maximally at 375–379 mμ (pH 8·3) and 365–367 mμ when NH_2OH was added to the extract (Table 2). These maxima are in accord with the data of Crescitelli and strongly suggest that retinene$_1$ is the chromophore of the photosensitive pigment of *Argyropelecus*. NH_2OH was added to a digitonin solution (pH 8·3) of crystalline all-trans retinene$_1$ to form the oxime, and the absorption spectrum of this preparation was measured (data of Dr Crescitelli). In Fig. 2 the points of this curve are plotted in scale for comparison with the NH_2OH difference spectrum. The close agreement of these curves is further evidence that retinene$_1$ oxime was formed in the NH_2OH experiment. The retinal pigment of *A. affinis* is a 478$_1$ photopigment. The subscript 1 means that retinene$_1$ is the chromophore of the pigment molecule.

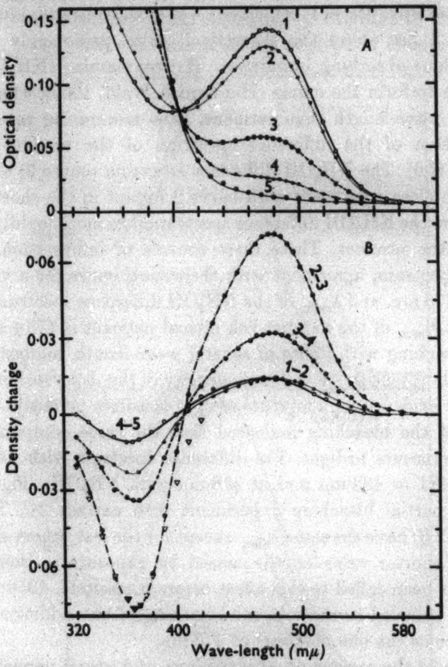

Fig. 2*A*. A partial bleaching experiment with *Argyropelecus* sample 2C (NH_2OH added). Curve 1, absorption spectrum of unbleached extract; curve 2, after 1 hr exposure to orange light (606 mμ); curve 3, after 90 min further exposure to yellow light (580 mμ); curve 4, after 30 min further exposure to green light (560 mμ); curve 5, after 10 min exposure to white light.

Fig. 2*B*. Difference spectra of experiment with sample 2C. Upward changes indicate loss of density; downward changes gain in density. Curve 1–2 is the result of the 606 mμ bleach; 2–3 the 580 mμ bleach, 3–4 the 560 mμ bleach, and 4–5 the white light bleach. The inverted triangles are the spectral densities for a digitonin solution (pH 8·3) of crystalline all-trans retinene$_1$ to which was added NH_2OH to yield the oxime. These points were scaled for plotting with the difference spectrum.

TABLE 2. Details of bleaching experiments

Species	Extract sample	Bleaching wave-length (mμ)	$\lambda_{\max.}$ of difference spectrum (mμ)	Density loss at maximum	$\lambda_{\max.}$ of product (mμ)	Density gain of product at maximum
Bathylagus wesethi	1 A	640	—*	—	—	—
		606	504	0·049	375	0·063
		606	497	0·047	373	0·070
		580	487	0·109	375	0·101
		580	483	0·101	376	0·082
		580	480	0·110	380	0·090
		W†	—‡	0·014	—‡	—
	1 B§	631	—‖	0·145	368	0·188
		606	498	0·108	368	0·081
		580	482	0·587	367	0·578
		580	480	0·122	367	0·098
		W	475	0·018	—‡	—
	1 C§	631	500	0·057	368	0·068
		606	497	0·075	365	0·080
		606	485	0·063	366	0·072
		580	480	0·133	367	0·144
		580	478	0·133	365	0·152
		560	478	0·114	367	0·110
		W	474	0·020	360	0·021
Argyropelecus affinis	2 B	640	482	0·013	—*	—
		606	483	0·060	379	0·012
		580	481	0·060	376	0·046
		580	482	0·082	375	0·072
		580	481	0·119	375	0·089
		W	474	0·030	—‖	0·038
	2 C§	606	480	0·014	365	0·023
		580	478	0·071	367	0·077
		560	478	0·032	367	0·034
		W	475	0·014	363	0·011
Sternoptyx obscura	3 A§	606	487	0·096	365	0·089
		580	484	0·209	367	0·216
		580	486	0·116	367	0·110
		560	484	0·100	368	0·093
	3 B	631	490	0·030	375	0·020
		606	485	0·067	378	0·053
		580	488	0·142	378	0·104
		580	487	0·154	375	0·114
		580	487	0·148	379	0·101
		W	385‡	0·056	—‡	0·004
Stomias atriventer	4 A§	631	—*	—	—	—
		606	491	0·013	363	0·047
		580	490	0·061	368	0·091‖
Lampanyctus mexicanus	5 A	631	500‖	0·059	373	0·060
		606	490‖	0·063	380	0·070
		580	490	0·348	378	0·275
		580	491	0·231	382	0·170
		560	492	0·096	377	0·066
	5 B§	631	—*	—	—	—
		580	490	0·093	367	0·170
		560	490	0·114	365	0·145
Melamphaes bispinosus	6 A	631	492	0·035	373	0·070
		580	490	0·320	375	0·266
		580	490	0·300	374	0·231
		580	490	0·197	374	0·151
		W	404	0·087	—‡	—

* Difference small, with no clear peak. † 60 W tungsten bulb used as bleaching light. ‡ Isomerization. § 0·1 ml. of 0·1 M-NH$_2$OH (pH 7·0) added to extract. ‖ Thermal instability of extract; difference spectrum not reliable.

A mixture of photosensitive pigments in a retinal extract of Bathylagus

A digitonin extract prepared from both eyes of four adult *Bathylagus wesethi* Bolin was analysed in the same manner as *Argyropelecus*. When corrected for the $D_{min.}:D_{max.}$ ratio, the absorption maximum of the unbleached pigment

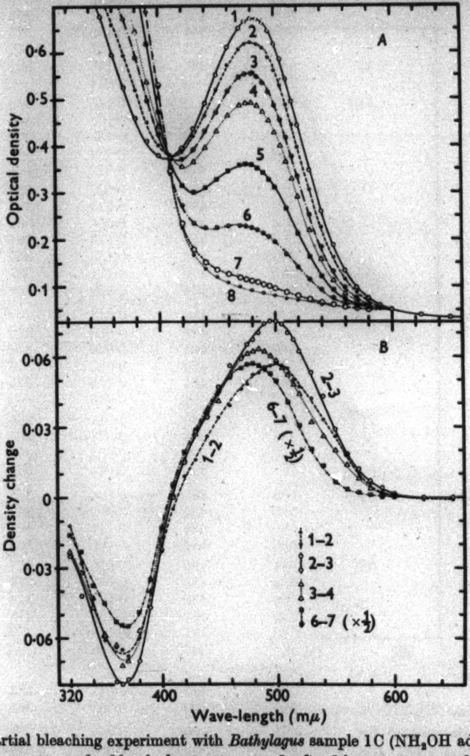

Fig. 3 *A*. A partial bleaching experiment with *Bathylagus* sample 1 C (NH_2OH added). Curve 1, absorption spectrum of unbleached extract; curve 2, after 1 hr exposure to red light (631 mμ); curve 3, after 30 min further exposure to orange light (606 mμ); curve 4, after 1 hr further exposure to 606 mμ light; curve 5, after 25 min further exposure to yellow light (580 mμ); curve 6, after 45 min further exposure to 580 mμ light; curve 7, after 40 min further exposure to green light (560 mμ); curve 8, after 10 min exposure to white light.

Fig. 3 *B*. Difference spectra of experiment with sample 1 C. Upward changes indicate loss of density; downward changes, increase in density. Curve 1–2 is the result of the 631 mμ bleach; curves 2–3 and 3–4, 606 mμ bleaches; and curve 6–7 (drawn to one-half scale), a 560 mμ bleach. Curves 4–5 and 5–6 (580 mμ bleaches) and 7–8 (white light bleach) are omitted for clarity of figure.

(Fig. 1*B*, curve 1) was estimated to be between 484 and 486 mμ. This curve does not coincide with the theoretical curve constructed from Dartnall's nomogram (curve 2), but is broader. This suggests what was later shown in partial bleaching experiments; namely, that two components were present in the extract and were summed in the absorption spectrum. The data of Table 2 show that bleaching with red light first removed a red-sensitive component ($\lambda_{\text{max.}} = 500$ mμ) and that with continued step-wise bleaches the difference spectra maxima gradually shifted to shorter wave-lengths, finally reaching 478 mμ. This information is shown graphically in Fig. 3, which gives the results of the experiment with sample 1C. In Fig. 3*B* the difference spectra show the selective bleaching of a rhodopsin-like component (curve 1–2, with $\lambda_{\text{max.}} =$ 500 mμ) by red light. Curves 2–3 (497 mμ), 3–4 (485 mμ) and 6–7 (478 mμ) gradually shift toward lower wave-lengths. Curves 4–5 (480 mμ) and 5–6 (478 mμ) are omitted for clarity of the figure. Curve 1–2 nearly fits the nomogram curve for a visual pigment with $\lambda_{\text{max.}} = 500$ mμ, and curve 6–7 is near the theoretical curve for $\lambda_{\text{max.}} = 478$ mμ. Both curves are slightly broader than the nomogram curve, indicating contamination of each with the other. The products of bleaching of the components were spectrally the same and resembled the bleaching product of *Argyropelecus*. Both pigments of *Bathylagus* are therefore believed to have retinene$_1$ as the chromophore. In Fig. 1*B* the difference spectra curve 4 (1–2 of Fig. 3) and curve 3 (6–7 of Fig. 3) are drawn in approximately the correct proportions to represent the total difference spectrum of the *Bathylagus* extract. Between 20 and 25% of the total bleaching was due to the 500$_1$ pigment and the remainder to the 478$_1$ photosensitive pigment.

Photosensitive pigments of Sternoptyx and Melamphaes

A retinal extract of another hatchet-fish, *Sternoptyx obscura* Garman, did not contain the 478$_1$ pigment. Instead, there was a 485$_1$ photosensitive pigment (see Tables 1 and 2, Fig. 4*A*). Partial bleaching experiments demonstrated the substantial homogeneity of this extract (possibly some red-sensitive pigment was present as a very small proportion of the whole). A whole-eye extract of the right eyes of sixteen *Melamphaes bispinosus* Gilbert contained a single light-sensitive pigment with $\lambda_{\text{max.}} = 488$ mμ. In this case the NH_2OH difference spectrum is lacking, owing to instability of the sample when NH_2OH was added. The retinal pigment, however, was shown to be homogeneous by a partial bleaching experiment (Table 2) without NH_2OH. The absorption spectrum (Fig. 4*B*) does not fit the theoretical curve for a visual pigment with $\lambda_{\text{max.}} = 488$ mμ. The slightly opalescent extract had an optical density of 0·050 at 700 mμ. Even after subtraction of 0·050 from all the readings, the gradually increasing absorption due to opalescence disturbed agreement with the nomogram curve as the short-wave end of the spectrum was approached. The difference spectrum without NH_2OH (curve 3) is also interesting, for it shows the distortion caused by the appearance of the bleaching product. The curve is narrower and shifted slightly toward longer wave-lengths; it is not as good an approximation of the true absorption spectrum as the NH_2OH difference spectrum. While the evidence for the 488 pigment of *Melamphaes* is not as precise as for the other pigments, Fig. 4 *B* is instructive in the methods used to locate $\lambda_{max.}$ of a photosensitive pigment of this type. As before, the product of bleaching (Table 2) suggested that retinene$_1$ is the chromophore of the pigment, which is a 488_1 mμ photosensitive pigment, location of the $\lambda_{max.}$ being within ± 2 mμ of this figure.

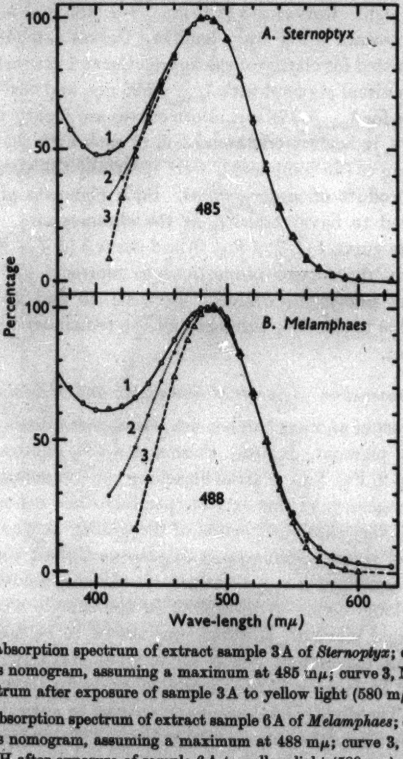

Fig. 4 *A*. Curve 1, absorption spectrum of extract sample 3 A of *Sternoptyx*; curve 2, constructed from Dartnall's nomogram, assuming a maximum at 485 mμ; curve 3, NH_2OH experiment, difference spectrum after exposure of sample 3 A to yellow light (580 mμ).

Fig. 4 *B*. Curve 1, absorption spectrum of extract sample 6 A of *Melamphaes*; curve 2, constructed from Dartnall's nomogram, assuming a maximum at 488 mμ; curve 3, difference spectrum without NH_2OH after exposure of sample 6 A to yellow light (580 mμ).

Difference spectra of Lampanyctus and Stomias

Two other species of deep-sea fishes were examined. The results suggested that both possess 490_1 retinal pigments. A whole-eye extract of the right eyes of sixteen adult *Lampanyctus mexicanus* Gilbert and a retinal extract of four large *Stomias atriventer* Garman both appeared to contain homogeneous light-sensitive pigments when subjected to partial bleaching experiments (Table 2). Since the extracts had high $D_{\text{min.}}:D_{\text{max.}}$ ratios (extract 5A of *Lampanyctus* excepted), greater reliance had to be placed upon the NH_2OH difference spectra. These are shown in Fig. 5 along with difference spectra of *Argyropelecus* and *Sternoptyx* for comparison. The products of bleaching were the same spectrally as in the other species examined. The photosensitive pigments of *Lampanyctus* and *Stomias* are tentatively considered 490_1 pigments.

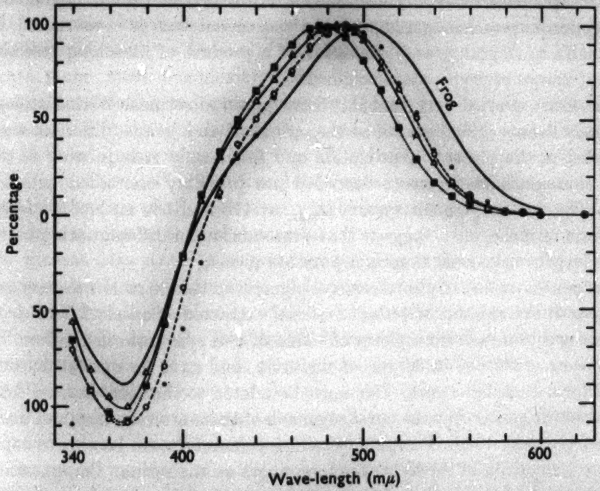

Fig. 5. Hydroxylamine difference spectra of deep-sea fish photosensitive pigments plotted as percentages of maximum density loss. ○, *Lampanyctus* sample 5B was bleached with 560 mμ light (maximum density loss 0·120). ●, *Stomias* (4A) was bleached with 560 mμ (maximum loss 0·066). △, *Sternoptyx* (3A) with 580 mμ (maximum loss 0·325), and ■, *Argyropelecus* (2C) with 580 mμ (maximum loss 0·071). The hydroxylamine difference spectrum of the frog *Rana pipiens* is also shown for comparison (data of Dr Crescitelli).

DISCUSSION

From the foregoing data it is clear that the technique of extraction and spectrophotometric analysis of the retinal pigments indicated general agreement with the results of Denton & Warren (1956) and of Walker (1956), who found in the conger a homogeneous visual pigment with $\lambda_{\text{max.}} = 487$ mμ. There was no one photosensitive pigment to be found in the six species of fishes examined, but rather a different situation in almost every species. All six, however, did have light-sensitive pigments with absorption maxima less than 500 mμ as Denton & Warren have stated (at least two of the genera were common to both studies). The six species of the present investigation represent three different orders of fishes and are not members of a single closely related group. *Bathylagus* belongs to one clupeoid subgroup and *Argyropelecus*, *Sternoptyx* and *Stomias* to another. *Lampanyctus* is a member of the Iniomi, and *Melamphaes* is a berycoid fish. Even in such an incomplete survey of deep-sea fishes as this, considerable diversity of retinal pigments is present.

Although no direct evidence is available that these retinal pigments of the bathypelagic fishes are visual pigments, they would appear to be such because of their photosensitivity, great concentration in the retina, characteristic absorption curves (compared with the theoretical curves constructed from Dartnall's nomogram), and generation of a product of bleaching resembling that of known retinene$_1$ visual pigments.

The fishes studied very probably have pure-rod retinae. With histological methods Brauer (1908) examined the retinae of each genus (different species) included in the present investigation and found only rods in each of them. The photosensitive pigments described are probably contained within the rods. The double pigment system ($\lambda_{\text{max.}}$ at 478 and 500 mμ) of *Bathylagus*, based on retinene$_1$ only, suggests that a mechanism for differentiating between wave-lengths may exist even in a pure-rod retina.

The concentration of photosensitive pigment in the extracts was very great, relative to the amount of retinal material extracted. Sample 2A of *Argyropelecus* was obtained from pieces of retina of two eyes from fish 4–5 cm long. They were extracted in 0·5 ml. of digitonin, and gave an optical density of 0·562 for a 1 cm light path. This must be related to the fact that the retinae have rods only and that the outer segments of these are very long and densely packed (Brauer, 1908). The photosensitive pigment should be able to capture a large proportion of the light which impinges on the retina. Computation of the photosensitive pigment density of the retina of deep-sea fishes would be valuable.

Photosensitivity of the retinal pigments

The actual light sensitivity of these pigments did not appear to be greatly different from that of rhodopsin. This was apparent from the length of time required to bleach the extracts with coloured light, which also has been employed with a number of rhodopsins (unpublished data). In a rough trial of this, an extract of frog (*Rana pipiens*) rhodopsin and the *Sternoptyx* extract 3C were simultaneously exposed to blue light (peak wave-length 493 mμ) about half way between their maxima of absorption. At intervals the extracts were removed from the bleaching apparatus and their absorption spectra were measured. When the percentage of the total bleaching was plotted against log. time, it appeared that the *Sternoptyx* extract may have been somewhat, but not greatly, more sensitive to the light used. This point of the relative photosensitivity of various visual pigments would be worth careful investigation, but at least as a first approximation the sensitivity of deep-sea fish eyes seems to depend on increased concentration of light-sensitive pigments more than on an increased photosensitivity of the pigments themselves.

Nomenclature

The nomenclature of visual pigments has not yet evolved into a uniformly applied system. The photosensitive pigments discussed in this paper need to be given names to identify them and, if possible, to describe their properties. Although the names 'rhodopsin' and 'porphyropsin' have often been rigidly applied to mean that there are only two rod visual pigments (e.g. Wald, 1955), recent investigations, as Dartnall (1957, pp. 38–41) has already pointed out, indicate much more complexity. 'Rhodopsin' and 'porphyropsin', when employed to describe rod visual pigments based respectively on retinene$_1$ ($\lambda_{\text{max.}} = ca.$ 500 mμ) and retinene$_2$ ($\lambda_{\text{max.}} = ca.$ 525 mμ), may have a certain value as broad group names; but they should not be used indiscriminately. For example, Crescitelli (1956) found that visual pigments of the geckos have spectral absorption characteristic of porphyropsin, but that the product of bleaching is similar to that of rhodopsin.

The present data strongly suggest that retinene$_1$ is the chromophore of the pigment molecule in all six species of deep-sea fishes examined. These results thus spread further the already wide limits to which the name 'rhodopsin' might possibly be applied. It is doubtful, however, whether 'rhodopsin' can be usefully stretched to include photosensitive pigments with $\lambda_{\text{max.}}$ ranging from 478 mμ (*Argyropelecus* and *Bathylagus*) to 524 mμ (geckos; Crescitelli, 1956). Qualification of the term 'rhodopsin', e.g. 'mammalian rhodopsin', is also inadmissible. Unpublished data of Dr Crescitelli show that the photosensitive retinal pigments of certain mammals approach those of the deep-sea fishes in wave-length of maximum absorption ($\lambda_{\text{max.}}$ of opossum 'rhodopsin' = 493 mμ). Since the deep-sea fish pigments are evidently based on retinene$_1$ also, it would be confusing to recognize them arbitrarily by a different name. For this reason 'chrysopsin' (Denton & Warren, 1956) has been avoided in the present paper. The photosensitive pigments of the deep-sea fishes have been designated instead by the wave-length of maximum absorption and the subscript 1 (to indicate that retinene$_1$ is the chromophore).

Biological significance

It was pointed out by Denton & Warren (1956) that $\lambda_{\text{max.}}$ of the retinal pigments of deep-sea fishes seems to be well suited for maximum sensitivity to those wave-lengths which are best transmitted by clear oceanic water. This statement was based upon the work of Jerlov (1951) during the Swedish Deep-Sea Expedition of 1947–48. Jerlov studied the effect of ocean water on light of different wave-lengths by lowering a photometer with coloured filters into the ocean and measuring the percentage transmission at increasing depths. His experiments show that $\lambda_{\text{max.}}$ of the transmitted light is about 475 mμ in oceanic waters. Several other workers had earlier studied the optical properties of water samples taken from various sources into the laboratory (James & Birge, 1938; Clarke & James, 1939; Hulburt, 1945). Their conclusions were similar to those of Jerlov. The work of James (later published as James & Birge, 1938) led Clarke as early as 1936 to write (p. 453): 'These results raise the question of the possibility of a shift in the sensitivity of the eye of a deep water fish toward the blue end of the spectrum.'

This apparent correlation between the $\lambda_{\text{max.}}$ of the retinal pigments of bathypelagic fishes and of the sunlight which reaches them suggests that the spectral absorption of the photosensitive pigments may be an adaptive characteristic, subject to natural selection. Bayliss, Lythgoe & Tansley (1936, p. 96) set out to 'inquire whether there is any correlation between the make-up of the retina of a given species and the depth which it normally frequents', but the methods then available for the investigation of retinal pigments were not sufficiently refined to establish any relation.

Barlow (1957), following a suggestion made by de Vries (1949), has proposed that the shift in maximum sensitivity of the eye toward the blue end of the spectrum at low levels of illumination (Purkinje shift) may have evolved as a means of obtaining a more favourable signal-to-noise ratio, thereby greatly increasing sensitivity of the eye to light. Such a shift in the spectral absorption of a visual pigment should be accompanied by a decreased thermal instability of the photochemical, producing the more favourable ratio. This suggestion offers an alternative explanation for the evolution of the blue-absorbing photopigments of deep-sea fishes. Fewer quanta might have to be absorbed by such more thermally stable pigments in order to give an unequivocal sensation of light (assuming the photosensitivity of these pigments to be approximately equal to that of 'rhodopsin'). This lowering of the visual threshold should be advantageous to a fish living in a low light-intensity environment. Perhaps the thermal stability factor has combined with the optical properties of oceanic water to exert a selective influence on $\lambda_{\text{max.}}$ of the deep-sea fish photopigments.

Recent studies (Clarke & Wertheim, 1956; Clarke & Backus, 1956) of submarine light intensities in the Atlantic Ocean show that bioluminescence, coming in frequent flashes, increases with increasing depths, equalling the intensity due to sunlight at 600 m. The spectral composition of luminescence in the sonic-scattering layer was studied at night (depth 100 m) by Kampa & Boden (1957), who found the average intensity maximum to be at 478 mμ, in the spectral region best transmitted by oceanic water. Two luminescent invertebrates, *Euphausia pacifica* and *Pyrosoma atlantica*, under laboratory conditions produced light with primary intensity maxima at 476 and 482 mμ, respectively, possibly indicating adaptation of the $\lambda_{\text{max.}}$ of luminescence to the spectral transmission of oceanic water. The close agreement of these values with $\lambda_{\text{max.}}$ of the bathypelagic fish pigments suggests that the photopigments may quite as likely have become adapted to bioluminescence as to the filtered sunlight available at moderate depths.

It should be remembered that even an initial survey of deep-sea fishes disclosed several pigments, with different peaks of absorption. Perhaps it is even possible that the eyes of a deep-sea fish are most sensitive to that light which its own species produces. Of the six species examined, all but *Bathylagus wesethi* and *Melamphaes bispinosus* have light organs. *Argyropelecus* has large tubular eyes (vertically directed), *Sternoptyx* and *Bathylagus* large normally shaped eyes, and the other fishes have somewhat smaller normal eyes. It would be interesting to compare the retinal pigments of these bathypelagic fishes, which live in or near the lighted zone and most of which may come to the surface at night, with the pigments of large-eyed, benthic rat-tail fishes (macrourids), which may never enter the photic zone.

SUMMARY

1. Photosensitive extracts were prepared from six species of bathypelagic fishes, representing several major taxonomic groups, by treating either whole retinae or the crushed eyes with 2% digitonin solution. The homogeneity of these extracts was tested by the method of partial bleaching.

2. Comparison of the spectral absorption of unbleached extracts, difference spectra without and with hydroxylamine, and the Dartnall (1953) nomogram established the spectral locations of the retinal pigments.

3. The maximum absorptions of these retinal pigments was below 500 mμ, indicating general agreement with the results of Denton & Warren (1956). But there was a range of values from 478 mμ in *Argyropelecus affinis* and in *Bathylagus wesethi* to 485 mμ in *Sternoptyx obscura*, 488 mμ in *Melamphaes bispinosus*, and 490 mμ in *Stomias atriventer* and *Lampanyctus mexicanus*.

4. In addition to a pigment at 478 mμ, the *Bathylagus* extract had a minor amount of a 500 mμ pigment.

5. The product of bleaching in all extracts was characteristic of that obtained with retinene$_1$ visual pigments. The spectral absorption of the hydroxylamine difference spectrum and of all-trans retinene$_1$ oxime were compared and suggest that the retinal pigments of these deep-sea fishes have the same chromophore as that in ordinary visual purple.

6. An attempt was made to correlate these findings with the spectral transmission of oceanic water, which has been studied by several workers. In clear oceanic water the maximum transmission is in the same spectral region as the maximum absorption of the retinal pigments. Recent reports of submarine measurements of the intensity and spectral composition of bioluminescence indicate that it is relatively important and that its spectral emission maximum coincides with the pigment absorption maxima. These facts suggest that the photopigments of deep-sea fishes may have become adapted to the light available at moderate depths in the ocean.

Dr F. Crescitelli and Dr B. W. Walker gave helpful advice and criticism during the course of the investigation. Much of the equipment used was made available by Dr Crescitelli's grant from the Division of Research Grants and Fellowships, U.S. Public Health Service. I am indebted to Mr A. W. Ebeling for identification of the fishes and assistance with systematics and to Dr G. L. Clarke, who critically read the manuscript. The crystalline retinene was kindly provided by Dr Grove Baxter of Distillation Products Industries.

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THE DISTRIBUTION OF CAROTID AND VERTEBRAL BLOOD IN THE BRAIN OF THE CAT

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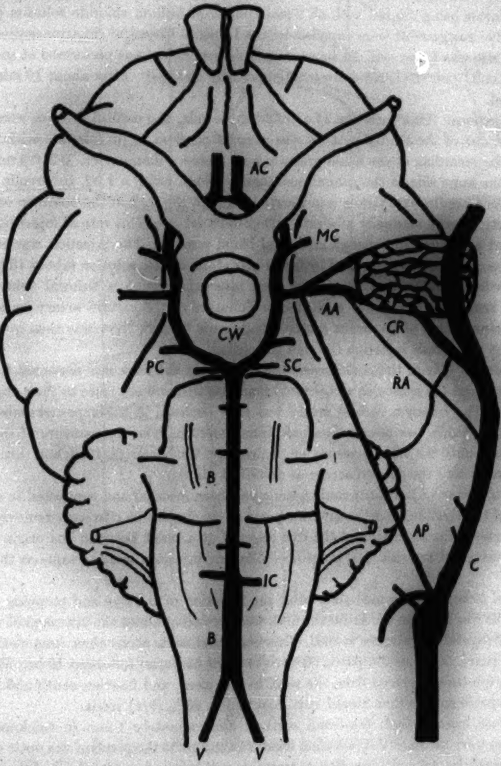
In animals commonly used in the laboratory the arteries supplying the blood to the brain may vary considerably from one species to another, especially in the contribution made to the circle of Willis by the vertebral and carotid arteries. In the cat the vertebral arteries unite near the anterior border of the foramen magnum to form the basilar artery. The basilar artery passes rostrally on the ventral surface of the brain stem to the upper border of the pons where it divides into the posterior cerebral arteries (Text-fig. 1). The carotid blood reaches the circle of Willis through anastomatic channels derived from the extracranial rete of the internal maxillary artery and via the ascending pharyngeal artery (Davis & Story, 1943; Chungcharoen, Daly, Neil & Schweitzer, 1952; Daniel, Dawes & Prichard, 1953).

It has generally been assumed that vertebral blood passes along the basilar artery to contribute to the circle of Willis, the brain stem thus being supplied with vertebral blood. However, it is equally possible that carotid blood may flow from the circle of Willis into the basilar artery. This problem cannot be decided by the study of the anatomical data alone. Physiological studies have been made in dogs and monkeys by Kramer (1912) using a dye injection technique and in rabbits by McDonald & Potter (1951), who made direct observations of the flow in the basilar artery. In both investigations it was concluded that carotid and vertebral blood met in the posterior communicating arteries of the circle of Willis.

The distribution of carotid and vertebral blood in the brain of the dog has been investigated in detail by Jewell & Verney (1957), who described a method based on the injection of dye suspensions into the blood stream. In the present investigation a similar method has been used to study the distribution of blood in the brain of the cat and also some direct observations have been made of flow in the basilar artery. It is often important to know what structures in the brain stem can be reached by blood from the carotid artery, and therefore special attention has been given to the determination of the caudal limit of tissue supplied by carotid blood. The effect on this distribution of heating the carotid blood has also been investigated, in view of the observation (Newman & Wolstencroft, 1956, 1957) that heating the carotid blood is followed by a fall of blood pressure in the intact and decerebrate cat, and that a similar fall of blood pressure can be obtained by direct heating of the medulla. Some preliminary results have already been reported (Holmes, Newman & Wolstencroft, 1957).



Text-fig. 1. Diagram of the circle of Willis (CW) and its communications in the cat. Afferent vessels carrying carotid blood are shown on the right side only. *AC*, *MC*, *PC*, anterior, middle and posterior cerebral arteries; *B*, basilar; *V*, vertebrals; *SC*, *IC*, superior and inferior cerebellar arteries; *AA*, anastomotic branches from the carotid rete, *CR*; *RA*, ramus anastomoticus; *AP*, ascending pharyngeal; *C*, carotid artery.

METHODS

Successful experiments were carried out on thirty-one cats of either sex, weighing between 4·0 and 6·0 kg, anaesthetized by intraperitoneal injection of sodium pentobarbitone (Nembutal, Abbott Laboratories Ltd., 45 mg/kg body wt.). The technique used for injection was similar to that described by Jewell & Verney (1957) and by Andersson & Jewell (1956). After a dye suspension had been injected into the artery whose field of distribution was to be investigated, the heart was stopped before recirculation had occurred, and sections of the brain were prepared and examined for the presence of injected material. Two dye suspensions recommended by Jewell & Verney (1957) were used. They were carbon black (11% solids) and Monastral Fast Blue (20% solids), the latter being diluted with an equal volume of sodium chloride solution (0·9%, w/v). Both these dye suspensions were supplied by the Imperial Chemical (Pharmaceuticals) Co. Ltd. The particle size was 1 μ or less. In some animals decerebration was performed at the level of the superior colliculi, with the carotid arteries temporarily clamped. After about 10 min the clamps were removed.

Carotid injections. After insertion of a tracheal cannula, the carotid arteries were exposed in the neck and one of the lingual arteries was cannulated close to its junction with the external carotid, for the recording of the blood pressure by a mercury manometer. 0·2–0·5 ml. of dye was injected on the same side as the cannulated lingual artery, using a 1 ml. tuberculin syringe and a no. 19 Record needle. The needle was inserted through the wall of the common carotid artery and the injection made manually at a rate of 0·02–0·04 ml./sec. This rate of injection was chosen because it did not cause any change in lingual blood pressure. The injection was continued for 10–15 sec; then the heart was stopped by injecting sodium citrate solution (about 10%) through a polythene catheter which had previously been inserted through a femoral vein and pushed towards the heart. In some later experiments puncture of the carotid artery by a needle was avoided by cannulation of its ramus muscularis dorsalis branch. Dye was then introduced into the carotid by retrograde injection through this artery.

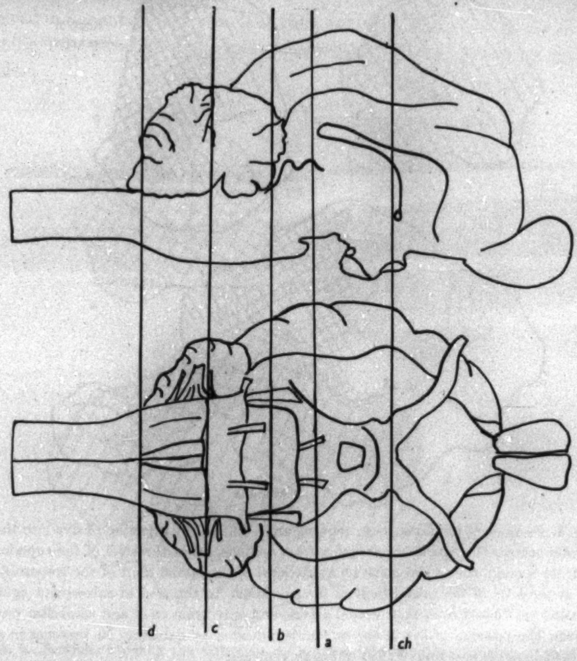
Carotid heating. One of the common carotid arteries in the neck was dissected free from surrounding tissue and the muscular and thyroid branches tied and cut close to their origin from the carotid trunk. The common carotid artery was then enclosed in a Perspex chamber, 3·5 cm in length, through which was pumped warmed saline solution. The temperature of the saline was gradually raised until the blood pressure fell (Newman & Wolstencroft, 1956) and then an injection of dye was made into the carotid artery as described above.

Vertebral injections. After a tracheal cannula had been inserted and connected to a respiration pump part of the sternum and the anterior parts of the first three ribs were removed. The right subclavian artery was exposed and the dye injected at a point between the origin of the right carotid and vertebral arteries. The procedure following injection was the same as that described above.

Histological techniques. Immediately after the injection of the dye and stopping of the heart a trephine hole was made in the skull; the neck was severed at about the 7th cervical vertebra and the atlanto-occipital membrane incised. The head and neck were then immersed in fixative (formalin 10 parts, acetic acid 5 parts, 50% (v/v) spirit 85 parts) for about 18 hr. The brain and spinal cord were then removed from the skull in one piece, and fixation continued for 2–3 days longer. The specimen was then stored until required in 96% (v/v) spirit.

After surface examination, free-hand sections approximately 1 mm in thickness were cut. Initially these were taken at the following levels (Text-fig. 2): the plane of the optic chiasma; the plane of the superior colliculi; junction of rostral two-thirds and caudal third of the pons; caudal limit of trapezoid body; and caudal limit of the pyramids. These sections were dehydrated in alcohol, cleared and mounted in balsam for examination with a binocular microscope. In most cases, further sections were made at intermediate levels.

Direct observation of the basilar artery in the anaesthetized animal. In these experiments the ventral surface of the brain stem was exposed. A length of trachea (including the larynx) and oesophagus was removed. The muscles attached to the occipital bone were removed and the bone itself cut away between the tympanic bullae, beginning at the junction with the atlanto-occipita membrane. It was considered inadvisable to extend the removal of bone as far as the rostra limit of the pons because of the bleeding that occurs when the bone in this region is cut. The meninges were cut and reflected and the basilar artery observed with the naked eye, or with a binocular dissecting microscope, during and after the injection of dye into the carotid or subclavian arteries. Instead of the dye suspensions mentioned above, a 1% solution of methylene blue was used in some experiments.

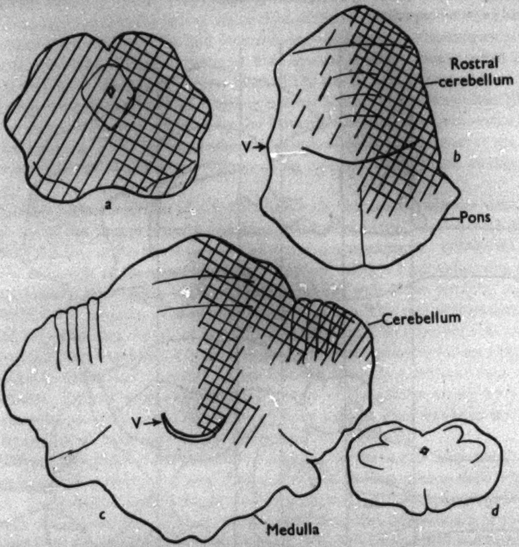


Text-fig. 2. Diagrams of the mid-sagittal and ventral surfaces of the cat brain, showing the levels of the main planes of section referred to in the text. (*ch*) plane of the optic chiasma; (*a*) plane of the superior colliculi; (*b*) pons; (*c*) caudal limit of trapezoid body; (*d*) caudal limit of pyramids.

RESULTS

Unilateral injection into a carotid artery

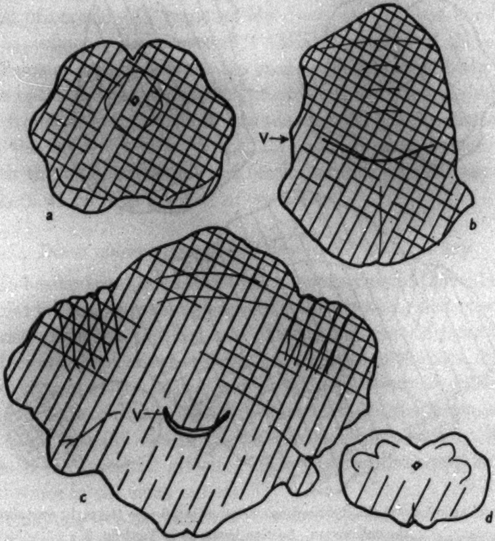
Following the injection of dye into the carotid artery of one side in ten cats, a naked-eye examination of the brain always showed pigment in some of the surface vessels. Pigment in the circle of Willis and its branches was often greater in amount on the side of the injection. Pigment was often also observed in the basilar artery and some of its main branches. On external examination the presence of dye in the minute vessels of the brain substance could be recognized by a greyish colour. This was always observed in the cerebral cortex and in parts of the cerebellar cortex, particularly dorsally on the side of the injection, but not in the medulla.



Text-fig. 3. Sections of the brain stem, showing areas reached by injection of dye into the right carotid artery. The figures are drawings from sections, (*a*) at the level of the superior colliculi; (*b*) through the caudal pons; (*c*) at the level of the caudal limit of the trapezoid body; (*d*) at the level of the caudal limit of the pyramids. In this and in subsequent figures, all sections are viewed from their caudal aspect, and only brain stem and cerebellar tissue are shown. The presence of dye in any section is indicated by hatching; its presence in greater amount by cross-hatching. *V*, line of fourth ventricle.

Examination of sections showed in all cases that the fine vessels of the cerebral hemispheres and thalamic region were well filled on the side of injection; on the opposite side some dye was present, but this was always less in amount. Passing caudally down the brain stem, the amount of pigment decreased. At the level of the superior colliculi the mid-brain was well injected, again more so on the side of the injection (Text-fig. 3*a*, Plate 1A). At the level of the caudal third of the pons (Text-fig. 3*b*), all specimens showed a considerable amount of pigment in the dorso-lateral zone or in the cerebellar peduncles, while the more ventral parts of the pons showed no pigment, or only a small amount, often confined to the larger vessels. In this plane the whole of the cerebellar tissue on the side of the injection usually showed some pigment; less was present on the opposite side.

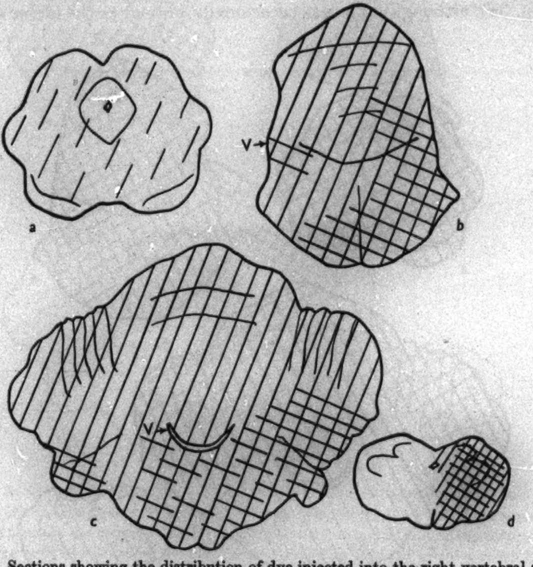
Sections at the level of the caudal limit of the trapezoid body showed little or no pigment in the medulla (Text-fig. 3*c*), and when present, this was usually confined to the larger vessels. The cerebellum showed some injection, especially in its dorsal and medial part. In no specimen of this series was pigment observed in fine medullary vessels at the level of the caudal end of the pyramids (Text-fig. 3*d*), although some was occasionally present in the larger superficial vessels.



Text-fig. 4. Sections showing the distribution following injection into the right carotid artery while blood in that vessel was being heated. Section levels as in Text-fig. 3.

Unilateral injection into a carotid artery while the carotid blood was heated on that side

In five preparations the distribution of pigment in the superficial vessels appeared to be more extensive than in the previous series. In the vessels of the rostral part of the brain bilateral distribution of pigment was now more marked, and the amount was often found to be equal in both thalami, irrespective of the side of injection. Cortical tissue, however, still usually showed some inequality. The other notable difference was the extension of pigment to a more caudal level. An appreciable amount was now found bilaterally at the level of the mid-brain (Text-fig. 4*a*, Plate 1B) and of the caudal end of the pons (Text-fig. 4*b*) and was not only confined to the dorsal region, although this was still often the most heavily injected part. Some pigment was observed in the medulla at the level of the trapezoid body (Text-fig. 4*c*), while in all cases except one pigment was present at the level of the most caudal extent of the pyramids (Text-fig. 4*d*). The cerebellum showed bilateral distribution of dye.



Text-fig. 5. Sections showing the distribution of dye injected into the right vertebral artery via the subclavian. Section levels as in Text-fig. 3.

Unilateral injection into a vertebral artery

Sections showed in one cat that vessels of the dorso-lateral regions of the cervical cord and lowest medulla were well filled on the injected side only (Text-fig. 5*d*). In the higher medulla and pons vessels were injected on both sides, and in the cerebellum widespread bilateral filling of fine vessels was observed, particularly in the more caudal and ventral zones (Text-fig. 5*c*). Above this level the amount of dye progressively diminished (Text-fig. 5*b*, *a*). The distribution in the cerebral cortex was limited to a sharply demarcated zone in the posterior part of the hemisphere and along the edges of the intercerebral fissure, i.e. in the probable field of supply of the posterior cerebral arteries; this zone was also filled in the carotid injections. Very little dye was present in other parts of the forebrain.

The results of the one experiment of this type were confirmed by the four experiments of the next series, except that in one of these dye injected into the vertebral artery did not reach any area above the level of the pons.

Simultaneous injections into carotid and vertebral arteries on the same side

A different coloured pigment was injected simultaneously into each vessel and the heart stopped as described under 'Methods'. In four cats the field of distribution of carotid and vertebral blood was as described above, but this group of experiments demonstrated a variable overlap of the carotid and vertebral fields of supply between the level of the trapezoid body below, and that of the optic chiasma above. The proportions of vertebral (blue) pigment decreased from below upwards in accordance with the descriptions already given. In the upper medulla, cerebellum and mid-brain, vessels which contained both the blue (vertebral) and black (carotid) pigment were found, as well as others which held pigment of one colour.

Direct observation of blood flow in the basilar artery

The main basilar trunk runs somewhat tortuously about the mid line. Under the binocular microscope pulsations could be clearly seen. Eight cats were used in this series. Dye was injected into one common carotid artery whilst the basilar artery was kept under observation. In three of the cats, dye appeared in the rostral part of the basilar artery, and was observed either to form a fluctuating boundary there, or to stream caudally to the origin of one of its lower branches, usually one of the inferior cerebellar vessels. No dye was seen to reach the vertebral arteries. These observations could be repeated a number of times in the same animal.

In the remaining five animals, however, dye injected into the carotid artery was not observed to reach the basilar, although it must be noted that the extreme rostral part of the latter had not been exposed.

When dye was injected into a vertebral artery, pigmented blood could be observed passing rostrally up the basilar artery and filling the inferior cerebellar and medullary vessels. The rostral limit of this flow varied, and in three out of four animals passed beyond the field of vision, presumably into the circle of Willis.

Streamline flow (confined to the side of injection) has been observed following both carotid and vertebral injections, but was more usual in the latter.

Carotid injection in the decerebrate animal

Examination of three specimens showed that the greater part of the circle of Willis was unaffected by the decerebration, and that anastomoses between it and the basilar artery remained intact. Sections of the brain stem after the injection of dye revealed a pattern of distribution similar to that of the normal animal.

DISCUSSION

The anatomy of the carotid vessels of the cat has been the subject of detailed examination in recent years (Davis & Story, 1943; Chungcharoen *et al.* 1952; Daniel *et al.* 1953). On most points the accounts of these workers are in agreement. The vessels supplying the rostral parts of the brain arise from the circle of Willis, which connects the basilar, superior cerebellar, posterior, middle and anterior cerebral arteries (Text-fig. 1). In the cat the internal carotid artery is not patent; the blood reaches the circle mainly by way of the anastomotic arteries which pass from the carotid rete via the orbital fissure into the cranial cavity (Text-fig. 1). In addition, a small ramus anastomoticus from the internal maxillary artery enters the skull via the foramen ovale, and a branch of this joins the anastomotic arteries. Another minor contribution is carried by the ascending pharyngeal, which enters the skull via the foramen lacerum medium, and joins the anastomotic artery where this passes through the dura. Daniel *et al.* (1953) state that there is an occipito-vertebral anastomosis, presumably of minor importance.

Our observations show that the middle and anterior cerebral vessels receive blood from the carotid arteries and not from the vertebrals, since dye injected into the carotids always reached the rostral parts of the cerebral cortex and dye injected into the vertebral arteries never did except in insignificant amounts. On the other hand, some dye injected into the vertebral arteries did reach the areas supplied by the superior cerebellar or posterior cerebral vessels, although the carotid seemed to be the main source of the blood flowing into these vessels.

The distribution of carotid and vertebral blood to the brain stem via branches of the basilar artery has been studied in several species. Both Kramer (1912), using dogs, and McDonald & Potter (1951), using rabbits, found that the carotid and vertebral blood streams met in the posterior communicating arteries of the circle of Willis so that the brain stem was supplied by vertebral blood. Jewell & Verney (1957), however, found that in some dogs carotid blood also reached the brain stem. They explained this in terms of the mixing of vertebral with carotid blood that had passed through the occipito-vertebral anastomosis, which is well developed in the dog (Jewell, 1952). Their evidence for this was that in one animal in which the occipital arteries had been tied no dye appeared in the brain stem after carotid injection.

Using a radiographic technique, Daniel *et al.* (1953) observed in the sheep that a considerable quantity of carotid blood entered the basilar artery under normal conditions. They also predicted, on anatomical grounds, a similar condition in the goat, and this was confirmed by Andersson & Jewell (1956), who showed that almost the entire brain of the goat is supplied by carotid blood. The basilar artery of the goat narrows as it is followed caudally, and its anastomoses with the vertebral vessels are small.

Examination of the basilar artery of the cat, both in plastic-injected specimens (Holmes, unpublished) and also by direct observation, shows a different condition. The two vertebral arteries unite to form the basilar which is considerably larger than either vertebral. When followed cranially, this gives off an inferior cerebellar branch on each side (which may be duplicated), either at the same or at different levels (Text-fig. 1). Rostral to this point the basilar artery narrows, and maintains approximately the same external dimensions for the rest of its course.

We have found that in the cat carotid blood reaches the dorsal part of the pons and cerebellum via the circle of Willis and superior cerebellar arteries, and may also reach medullary levels, apparently via branches of the basilar artery. The pattern of distribution rules out the possibility of the results being due to flow via an occipito-vertebral anastomosis. Vertebral blood supplies the greater part of the medulla, and may also pass rostrally as far as the superior cerebellar and posterior cerebral vessels.

Thus in the cat the junction of the carotid and vertebral blood apparently occurs in the upper end of the basilar artery or posterior part of the circle of Willis, and in some instances we were able to see the junction in the portion of the basilar artery that it was possible to expose. The position of the junction presumably varies with relative pressures and flows in the various vessels. It moves caudally if the vertebral artery is obstructed and in some animals after clamping the costo-cervical arteries near their origins. In those animals the costo-cervical arteries appear to make a significant contribution to the vertebral flow. Heating the carotid blood resulted in an enlargement of the field of supply of that vessel, apparently with the movement of the junction of carotid and vertebral blood flows towards the lower end of the basilar artery.

One objection to the dye method is the possibility of interference with the normal flow of blood. We consider it unlikely that this occurred in the present series of experiments; the distribution appeared to be the same whether the injection was made into the common carotid artery itself, or by a retrograde injection into one of its fine branches. Precautions were taken to prevent a rise in pressure during injection, although, surprisingly, direct observations of the basilar artery during intentionally high injection pressures showed no obvious difference in the point reached by the dye.

SUMMARY

1. The distribution of carotid and vertebral blood in the brain of the cat has been studied by the injection of dye suspensions in anaesthetized animals followed by histological examination.

2. Carotid blood supplies the cerebral hemispheres and thalamic regions. It also contributes to the blood supply of the cerebellum, pons and medulla, perhaps as low as the caudal limit of the trapezoid body. In nearly all specimens the vessels were better filled with dye on the side of injection.

3. Vertebral blood supplies medullary, pontine and cerebellar tissue. The vertebral and carotid fields overlap in the upper brain stem and cerebellum.

4. In some of the animals direct observation has shown that blood may flow caudally in the upper part of the basilar artery, so that carotid blood may reach lower branches. In other instances carotid blood did not extend below the upper end of the basilar artery into the available field of vision.

5. The point of junction of carotid and vertebral blood is variable, but is usually in the upper end of the basilar artery or in the posterior communicating arteries of the circle of Willis close to the basilar artery.

6. Decerebration, by the method employed, does not change the pattern of distribution of carotid blood in the brain stem and cerebellum.

7. Caudal extension of the carotid stream is favoured by heating the carotid blood or by any factor which diminishes the vertebral flow.

We thank Messrs Imperial Chemical (Pharmaceuticals) Ltd., for the gift of dye suspension.

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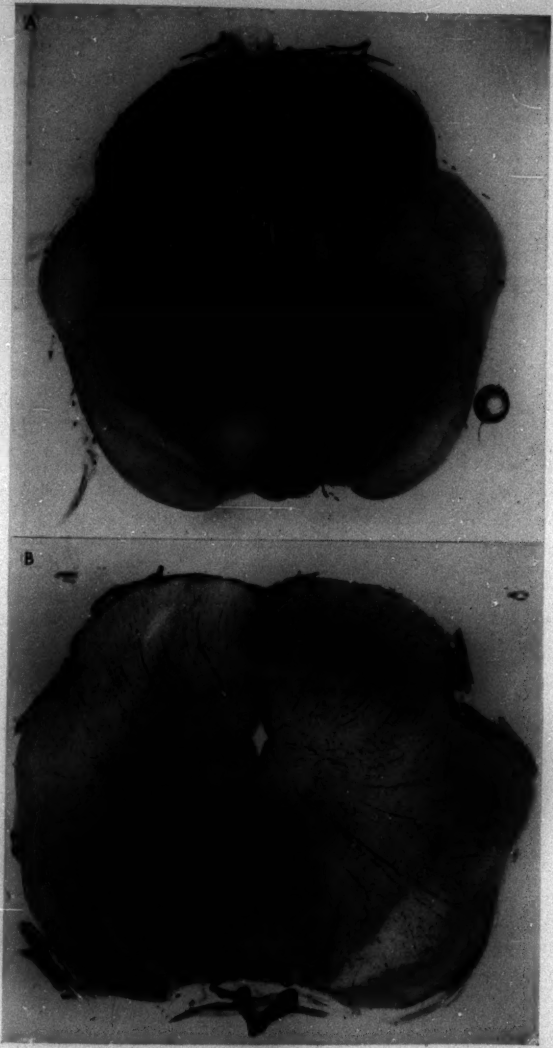

Photographs of sections at the level of the superior colliculi viewed from their caudal aspect A shows the distribution following injection of dye into the right carotid artery. B shows the distribution when the dye is injected during heating of the blood in the right carotid artery.

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THE SOURCES OF SLOW ELECTRICAL ACTIVITY IN THE FROG'S RETINA

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In three earlier papers (Brindley, 1956*a*, *b*, *c*) in which slow electrical responses of the excised eye of the frog to illumination were investigated, the following tentative conclusions were drawn:

(i) The layer of high electrical resistance and capacity (R membrane), which a micro-electrode penetrates when it is about 230μ from the inner boundary of the retina, is the external limiting membrane.

(ii) A complex sequence of responses to diffuse illumination of the whole retina, recorded by a micro-electrode advanced in steps through the retina, similar to that described by Tomita (1950), is a normal phenomenon. A simple sequence of responses (Ottoson & Svaetichin, 1953; Brindley, 1956*c*, Fig. 6) is found in retinae which have lost some, but not all, of their normal functions.

(iii) The electroretinogram (excluding its c-wave, for which no evidence is available) is entirely or almost entirely generated by the rods and cones.

The present paper presents new evidence which supports these three conclusions, and also provides some indication of the sources of the electrical activity which is present in those retinae which give the complex (Tomita, 1950) pattern of responses and absent in those which give the simple (Ottoson–Svaetichin, 1953) pattern.

METHODS

These were mainly those described in Brindley (1956*a*). Opened excised eyes of *Rana temporaria* were used wherever the contrary is not stated. The following features are new:

Preparation. In a few experiments pithed frogs were used, the cornea, iris and lens of one eye being removed, the body of the frog attached to the horizontal micromanipulator with the opened eye facing upwards, and the micro-electrode lowered into the retina in the usual way. The 'indifferent' electrode was placed in the mouth; with this precaution, only a small fraction of the electrocardiogram appeared on the record.

Amplifier. The directly-coupled amplifier shown in Fig. 1 was used. The 'cascode' arrangement of the first two valves is familiar in communication engineering, where its principal advantage is in minimizing shot noise (Valley & Wallman, 1948). It does not seem to have been used in electrophysiology, where, besides the low noise, it has the advantages of great simplicity and very satisfactory control of grid current. With the present circuit the grid current taken by V_1 could, by altering the potential of the grid of V_2, be varied from $+3 \times 10^{-9}$ A through zero to -2×10^{-11} A without substantial change in gain.

In all records the beginning of the stimulus is marked by a downward and its end by an upward artifact. Its duration is always 0·63 sec.

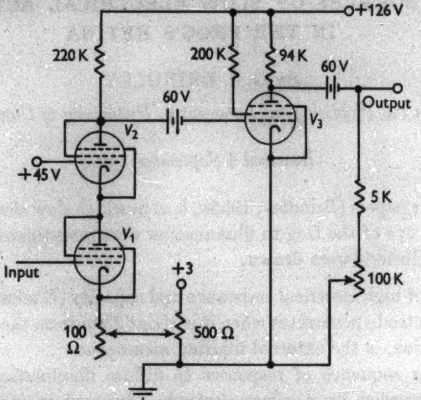

Fig. 1. Circuit-diagram of preamplifier. V_1 and V_2 are Mullard EF 37 A. V_3 is Brimar 6 BR 7. The coupling batteries are pairs of 'Ever-Ready' B 123 hearing aid batteries. The variable cathode load of the input stage serves as a fine balance-control.

RESULTS

The nature of the R membrane

Presence of an R membrane in mammalian eyes

Fig. 2 shows the result of passing square pulses of current between the vitreous and sclera of an opened excised rat's eye, and recording the resulting potential pulses between a large electrode in contact with the sclera and a micro-electrode inserted into the retina from its inner surface, as described earlier for the frog (Brindley, 1956*a*, 1957*a*). The records are very similar to those obtained with frogs' eyes (see Brindley, 1957*a*, Fig. 2), and indicate the presence of a structure of high electrical resistance and capacity ('R membrane') which the electrode penetrates between 250 and 300μ from the inner surface of the retina as it is advanced. Closely similar records were obtained from seven of twelve other eyes tested, the R membrane being sometimes penetrated on the same step in withdrawal as in advancement, sometimes 50μ nearer inner surface. The lack of success in five experiments, in which the potential pulses were very nearly square when the micro-electrode was in the vitreous, and did not change suddenly at any point within the retina, may well be attributable to mechanical injury to the retina during dissection. This is more difficult to avoid in the rat than in the frog, because the rat's sclera has very little rigidity, and the eye is apt to collapse when opened.

For the experiment of Fig. 2, the change in height of pulse between the inner surface of the retina and the point immediately before the R membrane was reached corresponds to a resistance of 89 Ω cm^2, and that on crossing the R membrane to 99 Ω cm^2. The mean values of these quantities for all the eight successful experiments were 72 and 112 Ω cm^2. These are very similar to the corresponding quantities for the frog (97 and 100 Ω cm^2).

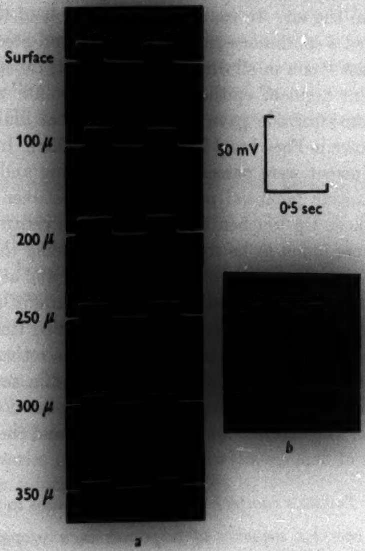

Fig. 2. (*a*) Potential pulses recorded between a micro-electrode, inserted in steps of 50 µ into a rat's retina from its inner surface, and a large electrode in contact with the sclera, when square pulses of current of 16·8 µA were passed between the sclera and vitreous. (*b*) Potential pulses recorded between electrodes in the vitreous and in contact with the sclera of a rabbit's eye when square pulses of current of 61 µA were passed between the sclera and vitreous. Above, retina intact; below, after removing the retina.

Fig. 2 also shows the potential pulses recorded by electrodes in contact with the vitreous and sclera of a rabbit's eye on passing square pulses of current between a second similar pair of electrodes. As with the frog and rat, the pulses recorded with the retina intact are rounded, as if there is a capacity as well as a resistance between the electrodes. When the retina is removed, this rounding is lost, and the size of pulse diminished by an amount corresponding to a resistance of 154 Ω cm².

Absence of an R membrane from isolated retinae

The retinae were removed from freshly excised eyes of six fully dark-adapted frogs. Each retina was mounted with the receptors uppermost on a dome of porous earthenware soaked with Ringer's solution, similar in size and shape to the vitreous body of an average frog. A ring, 1·5 mm in diameter, of fine silver wire was gently lowered on to the surface of the retina, upon which it rested, touching along its whole circumference. The upper surface of the retina remained exposed to the air. In each retina inspection of the surface with a microscope revealed a continuous pattern of purple and green rods within the ring and for at least 1 mm in all directions outside it, except for a few small patches of adherent pigment epithelium; and all points within this region tested with a micro-electrode gave responses to local illumination roughly similar to those shown in Figs. 1 and 2 of Brindley (1956*c*). In all retinae, when square pulses of current were passed between the ring and the earthenware dome and the resulting potential pulses recorded between the dome and a micro-electrode whose tip touched the retina at the centre of the ring, the potential pulses were found to be square. On advancing the micro-electrode through the retina they decreased only slightly in size, and at no point changed in shape. One retina was fixed and embedded in paraffin at the end of the experiment. Examination of stained sections confirmed that rods and cones were present over the whole of the central part of the retina, from which the electrical records were taken, though they were bent in several regions and obviously broken in a few, and many of the rods appeared to have lost their extreme outer ends. The external limiting membrane and the layers in front of it showed no signs of damage anywhere.

Normality of Tomita's complex sequence of responses to illumination

Persistence of the complex sequence of responses in a preparation with intact blood supply

In excised eyes the complex sequence of responses to uniform illumination of the whole retina is usually obtained for the first half-hour or so after excision. Thereafter the complexities diminish and then disappear, leaving an eye in which a typical normal electroretinogram can be recorded from all points in front of the R membrane, and little or no response to illumination from points behind it. With further lapse of time, the electroretinogram itself diminishes in size and finally disappears. Thorough removal of the vitreous humour, which should assist the exchange of oxygen and carbon dioxide between the retina and the atmosphere, usually delays the disappearance of the complexities, and also the final disappearance of the electroretinogram. These observations suggest that the complexities are a normal phenomenon, and their disappearance a sign of deterioration, perhaps due to lack of oxygen or poisoning with carbon dioxide. It might, however, be objected that they could result from a disturbance of the retina caused by removing and opening the eye, a disturbance from which it recovers in about half an hour. This objection is made much less plausible by the fact that in pithed frogs, in which the retina continues to be supplied with blood, the complexities may last very much longer. The pithed frog is not a wholly satisfactory preparation, for in some, perhaps those with a relatively high blood pressure, approaching that of an intact frog, the cut edge of the choroid bleeds profusely and covers the anterior surface of the retina with clot, and in others, thought to be those with an unusually low blood pressure, the retina lasts no longer than that of an excised eye. However, from one pithed frog typical complex responses were recorded without substantial change during a period of 7·5 hr, and from three others for periods exceeding 2·5 hr. No excised eye ever gave complex responses for more than 70 min.

Evidence that the complexities are not caused by the presence of the electrode

The region from which a micro-electrode is recording might be atypical because the electrode casts a shadow on it, because potassium chloride solution leaks from the tip, because the grid current taken by the input valve of the amplifier flows through it, or because it is mechanically deformed by the insertion of the electrode. Against these possibilities we have the following arguments:

(i) If a grating having 25 lines/cm is used as stimulus instead of a uniform field of light, it makes almost no difference to the response whether a line or a space falls on the electrode (see Fig. 3).

(ii) The complex series of responses can be recorded with an electrode filled with Ringer's solution (Tomita, 1950) or with silver wire (Tomita & Funaishi, 1952).

(iii) Variations of grid current from −24 pA through zero to +530 pA have no effect on the responses (see Fig. 3).

(iv) Almost the same sequence of responses is obtained on withdrawing the electrode as on advancing it, although the mechanical deformations must be very different.

Origin of the electroretinogram

The possibility of obtaining the simple (Ottoson–Svaetichin) pattern of responses at all points of a retina

It was earlier observed (Brindley, 1956*c*) that when the complex sequence of responses has disappeared from one region of a retina it is usually found to be absent also from other regions, even though the electroretinogram may be completely normal. Tomita & Torihama (1956) seem to have observed the same phenomenon, for they write: 'The focal potentials are very susceptible to ageing at higher temperatures. At 17° C and above they usually disappear in 10 minutes or less, while the e.r.g. does not change much,' and later: 'Once the focal potentials have disappeared at one site, punctures of other regions of the same retina usually fail to detect them, or if detected, they are very feeble.'

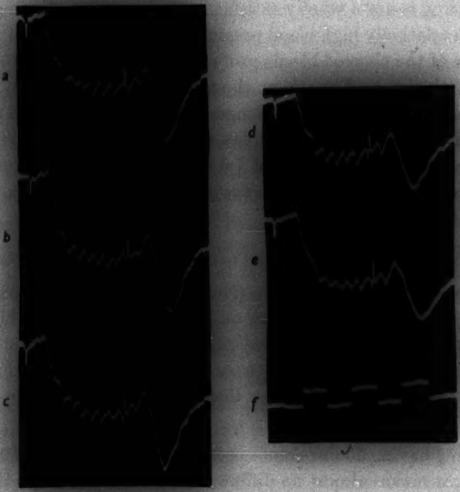

Fig. 3. Left: responses to uniform illumination at 43 lm/m², recorded with a micro-electrode inserted from the inner surface of the retina of an excised frog's eye to a depth of 160μ, the amplifier drawing a grid-current of (*a*) +530pA, (*b*) +1pA, (*c*) −24pA. Right: responses recorded from the same point on the retina, the stimulus being a grating of equal lines and spaces having 25 lines/cm: (*d*) centre of a dark line falling on the electrode; (*e*) centre of a space falling on electrode. (*f*) 154μV.

The following experiment was done to verify that it is possible to obtain the simple (Ottoson–Svaetichin) pattern of responses at all points of a retina which gives an e.r.g. of normal shape and nearly normal size. From each of six opened excised eyes the anterior parts of the sclera, choroid and retina were cut away with scissors, so that the posterior pole which remained formed only about a one-sixth segment of a sphere, and hence, when placed in the apparatus, was wholly accessible to the micro-electrode, which could enter the retina at any point without great obliquity. The preparation was kept in the apparatus at about 18° C for 25 min. Then a number of points scattered all over the retina were tested, responses to illumination being recorded at the surface of the retina and at depths of 100, 200 and 300μ. In the two best of these six experiments 25 and 21 points were tested, and all gave the simple pattern of responses, though the e.r.g. was of normal shape, and its amplitude was about 400μV at the beginning of the experiment and about 200μV at the end.

The effect of intensity of stimulus on the complex (Tomita) pattern of responses

Fig. 4 shows the responses to spatially uniform stimuli at three intensities, recorded at the inner surface of a retina and at five depths within it, all stimuli being superimposed on a steady background ten times less bright. The effect of increasing intensity on the electroretinogram (surface record) is simply to increase its amplitude and shorten its latency and time course. Opposite effects, or effects different in kind from these, were never found in any range of intensities. The responses recorded from within the retina, on the contrary, may increase in amplitude and shorten in time course with increasing intensity in one range of intensities, but decrease and lengthen in another, as is seen at 200μ in Fig. 4; and they may show features, such as the diphasic off-effect at 100μ in Fig. 4, which are conspicuous at some intensities and wholly absent at others.

Additivity in the electroretinogram

It was shown earlier (Brindley, 1956*b*) that for fields of 0·5 mm^2 area and over, if the effects of stray light are eliminated by superimposing the test flash on a steady background, the e.r.g. obtained on illuminating any region of an opened excised frog's eye is the sum of the responses obtained on illuminating the parts of that region separately. This sufficed to allow the large departures from additivity at comparable field sizes, found by Adrian & Matthews (1927) in the *absence* of steady background, to be attributed to stray light, and hence discarded as evidence concerning the origin of the e.r.g. However, the range of field sizes concerned was one in which even the local responses recorded intraretinally were almost additive, so that the finding of additivity in the e.r.g. provided no substantial evidence that these local responses do not contribute to it.

Improvements in the electrodes and amplifier, and the device of using multiple fields, have now made it possible to test for additivity of the e.r.g. with fields small enough for the local responses recorded intraretinally to show very large departures from additivity (see Fig. 9 of Brindley, 1956*c*; the degree of non-additivity seen there is typical of that found in many similar experiments). For the e.r.g., no departures from additivity were found. In particular, a single circular field of area 2·08 mm^2 was found consistently to give the same response as a field of practically the same total area (2·02 mm^2) made up of 24 small circles, each about 0·3 mm from its neighbours. Fields made up of three circles each of area 0·19 or 0·96 mm^2 gave responses indistinguishable from the sums of the responses to their components applied separately. The response to three circles each of area 0·96 mm^2 was indistinguishable from a 0·96/0·19 times multiple of the response to three circles each of area 0·19 mm^2. Typical records, and photographs of the fields with which they were obtained, are shown in Figs. 1*a* and 1*b* of Brindley (1957*b*).

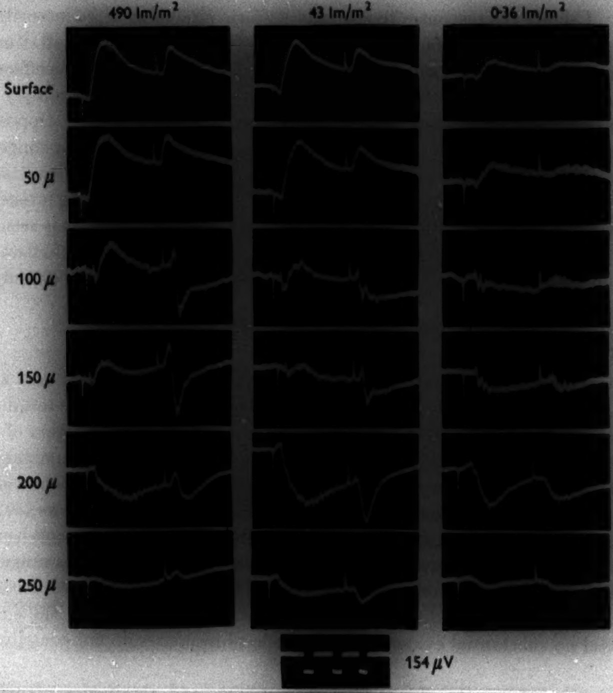

Fig. 4. Effect of varying the intensity of the stimulus on the e.r.g. and on the responses to uniform illumination of the retina, recorded with a micro-electrode inserted into it from the inner surface.

Proportionality of response to stimulus at very low intensities

If the e.r.g. is generated by structures, such as the rods and cones, which may be expected to respond independently of each other, the electroretinographic response to a short flash should be proportional to its intensity for flashes weak enough to provide an average of substantially less than one quantum absorbed per receptor. It has been found impossible to test this inference in the frog. The weakest short (8 msec) flash which gave a clearly measurable response (of about $10\mu V$) was 8×10^{-5} lumens × seconds/m^2 at wave-length 520 mμ, or about 4×10^{-5} quanta/mm^2 incident. Inferring from the data of Denton & Wyllie (1955) that there are about 3×10^4 rods/mm^2 in *Rana temporaria* and that about 65 % of incident light at wave-length 520 mμ is absorbed in rhodopsin, this corresponds to 8·6 quanta absorbed per rod.

This result, though disappointing in itself, does suggest that, given a preparation a little more stable and sensitive than the excised frog's eye and an efficient means of improving the signal-to-noise ratio by averaging many similar responses, the e.r.g. might be detected for flashes providing an average of less than one quantum absorbed per receptor, and the inference of proportionality be tested.

DISCUSSION

The nature of the R membrane

The presence of an R membrane in mammalian eyes shows only that it is not a structure peculiar to the frog or to amphibia. It is consistent with the hypothesis that it is the external limiting membrane, without giving that hypothesis any strong support.

The absence of an R membrane in isolated retinae, the whole relevant region of which had an intact pattern of rods as far as could be seen by microscopical examination before the experiment, gave electrical responses to light everywhere, and appeared on subsequent examination of stained sections to be undamaged except for many bent and a few evidently broken receptors, provides fairly good evidence that the R membrane is not a structure lying in front of the rods and cones. In removing the retina from an opened dark-adapted frog's eye only the outer surface is exposed to direct risk of mechanical injury, and the presence of an intact pattern of rods makes it very unlikely that any such injury can have extended to structures internal to them and protected by them. This argument, and the histological appearance in the one retina of which sections were cut after the experiment, indicate that the external limiting membrane itself probably remained undamaged in these experiments. This can readily be reconciled with the hypothesis of the identity of the R membrane and the external limiting membrane if it is assumed that the surface membranes of the rods and cones act as extensions of the R membrane, accounting for its otherwise astonishingly high capacity of about $100\mu F/cm^2$; rupture of the surface membranes of any substantial number of receptors (which almost certainly occurs) will short-circuit the R membrane so that its effect on the distribution of potential across the retina disappears.

If, as here suggested, the surface membrane of the rods and cones, as well as the external limiting membrane itself, forms part of the R membrane, the event electrically identified as the crossing of the R membrane by a micro-electrode inserted into the retina from its inner surface may not always represent the penetration of the external limiting membrane by the electrode; sometimes the tip of the electrode may be within a rod or cone when it reaches the level of the external limiting membrane, so that the electrical barrier is not crossed until later, when it leaves the rod or cone. When neighbouring regions of one retina were tested, the apparent depth of the R membrane from the inner surface of the retina did not often vary from one impalement to another by more than about 30μ. This suggests either that such penetration of the level of the external limiting membrane within a rod or cone is uncommon, or that when it happens the electrode usually leaves the rod or cone in which it lay, or disrupts its surface membrane, before it has travelled more than about 30μ beyond the external limiting membrane.

Failure to detect an R membrane in isolated retinae does not of course provide evidence against the hypothesis that it is some structure *behind* the external limiting membrane, for example, the pigment epithelium. This hypothesis, however, is made very unlikely by the measurements of its distance from the anterior surface of the retina and from the sclera (Brindley, 1956*a*). Further evidence that the R membrane is not the pigment epithelium is that in the simple pattern of response the e.r.g. appears across the R membrane, and hence must be generated by structures which penetrate it; for origin of the e.r.g. from the pigment epithelium can only with great difficulty be reconciled with the close similarity between the spectral sensitivity curve of the e.r.g. and the absorption spectrum of rhodopsin (Chaffee & Hampson, 1924; Granit & Munsterhjelm, 1937), and with the Purkinje shift which the e.r.g. shows on light-adaptation (Granit & Wrede, 1937).

The source of slow electrical activity in the frog's retina

The possibility of sampling as many as 25 points scattered all over a preparation and finding the simple (Ottoson–Svaetichin) pattern of responses at all of them, though the e.r.g. is completely normal in shape and of not much less than normal size, provides very strong evidence that the intraretinal responses of the complex (Tomita) pattern make no contribution to the e.r.g., or at least none that substantially affects its shape. The simplicity of the effect of light intensity on the e.r.g. and the complexity of its effect on the intraretinal responses of the Tomita pattern provide further support for the same conclusion. In an eye which gives the Ottoson–Svaetichin pattern of responses everywhere, the R membrane (which we will take to be the external limiting membrane) is polarized with the time course of the e.r.g. The structures which carry the current to do this must either all carry current with the same distribution in time, or, if they are of several kinds in this respect, the carriers of any one temporal distribution of current must have on the average the same spatial distribution of sources and sinks as those of any other temporal distribution of current; for if not, the response would, contrary to observation, differ in shape for different depths of the recording micro-electrode within the retina.

It was earlier inferred (Brindley, 1956*c*) that carriers of current across the R membrane must necessarily be rods and cones. This inference would be valid for mammalian eyes; but it was made for the frog in ignorance of Landolt's discovery (Landolt, 1871; Cajal, 1894, p. 72 and plate 2) that a large proportion of the frog's bipolar cells have processes which penetrate the external limiting membrane, and could possibly carry current across it. We must also consider, in discussing the structures which carry current across the external limiting membrane, that the potential developed across this membrane, though it may, in the simple pattern of response, be everywhere identical with the e.r.g., may also, in the complex pattern of response, be very different. It was earlier suggested (Brindley, 1956*c*) that the elements active in the complex and inactive in the simple pattern of response might fail to contribute to the e.r.g. because they were oriented tangentially in the retina. If this were the whole explanation of their failure to contribute, the potential developed across the external limiting membrane, much though it might differ from the e.r.g. at any one point investigated, would necessarily be equal to it when averaged over the whole retina. This cannot be so; for in the present experiments, as apparently also in Tomita's work, complex patterns closely resembling those shown in Fig. 7 of Tomita (1950) or Fig. 8 of Brindley (1956*c*) were consistently found at all points of freshly dissected retinae in good condition. Examples of the variability from one eye to another of the complex response patterns and the effect of the intensity of the stimulus on them are shown in Fig. 5. The variability from point to point in the same retina was commonly even less, though the oscillations at about 12/sec often differed in phase and sometimes in frequency between points remote from each other. It is clear that the average of such responses over many points will not differ very much from a sample taken at any one point, though the oscillations are likely to be absent from the average. If the structures which carry the current responsible for the e.r.g. in the Ottoson–Svaetichin state do so also in the Tomita state, in which its time course is the same, there must in the Tomita state be other structures which carry across the external limiting membrane current of time course corresponding (in the sense of being approximately its derivative with respect to time) to the difference between the potential across the membrane and the electroretinographic potential. The carriers of this current should, on the evidence of the spatial distribution of potential in front of the membrane, extend much closer to the inner surface of the retina than the structures which carry the current responsible for the e.r.g. Only the rods and cones are suited anatomically to carry the electroretinographic current, and only the bipolar cells to carry that which corresponds to the difference between the e.r.g. and the potential across the external limiting membrane.

There remains on this hypothesis the problem of why the latter current fails to leave any substantial trace in the e.r.g. A possible explanation, consistent with all the experimental results, is that the bipolar cells act as symmetrical tripoles, a sink lying between two sources. If they are symmetrical in terms of potential, they must be very asymmetrical in terms of current, since the external limiting membrane has a much higher radial resistance than the anterior layers of the retina.

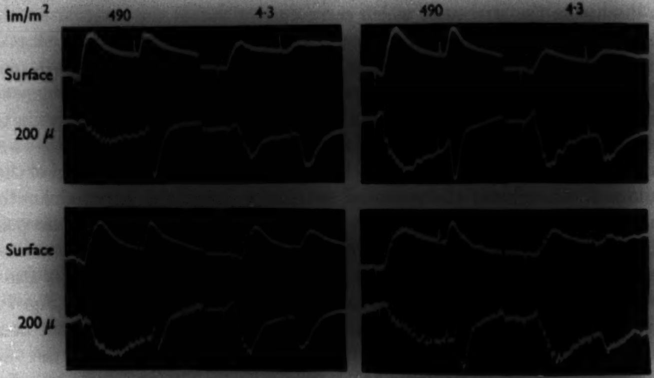

Fig. 5. Responses of four frogs' retinae to uniform illumination at 490 and 4·3 lm/m². In each group, the upper two records are electroretinograms, i.e. responses recorded with the micro-electrode at the inner surface of the retina, and the lower two are the responses to the same stimuli recorded with the micro-electrode at a depth of 200 μ. All records were taken within 15 min of killing the frogs.

The hypothesis of two kinds only of current-carrying conductors—the rods and cones on the one hand, carrying current of time course corresponding to the e.r.g., and the bipolar cells on the other, carrying, as symmetrical tripoles, current of time course corresponding to the difference between the e.r.g. and the potential across the external limiting membrane—accounts very well for the main features of both the simple and the complex patterns of intraretinally recorded responses to spatially uniform illumination. It leaves the origin of the oscillations at about 12/sec uncertain, for they could equally well be a detail of the activity of the bipolar cells or be due to tangentially orientated elements; and there is one feature which it fails to explain, namely that the off-effects at intermediate depths in the neural layers of the retina are often such as cannot be constructed from any sum of multiples of that at the surface of the retina and that immediately in front of the R membrane, or not from the same sum of multiples as is required to construct the on-effect. This is clearly shown in the records for 490 and 43 lm/m^2 in Fig. 4. Either the internal longitudinal current must differ in time course in different parts of a bipolar cell, or other cells must make some contribution. It is clear that the origins of slow changes of potential within the retina are complex enough to be extremely difficult to analyse with certainty. If two or even three different kinds of simple current-carriers without interaction between one region of the retina and another had been found sufficient to explain all the present results, it might have been possible, after testing a few more consequences of the hypotheses required, to become reasonably sure that they accounted for the whole electrical behaviour of the system; but three kinds of simple carrier are insufficient, for structures resembling the horizontal cells are required to account for the positive-going responses of Fig. 4 of Brindley (1956*c*), and these are inappropriate to explain the change in shape of off-effects with depth. At least four simple carriers or two simple and one complex are needed, and the number of possible explanations of any result is still further increased by the knowledge that one region of the retina certainly interacts with another. The fortunate chance that the complexities can disappear as the preparation ages without change in the e.r.g. allows us to be fairly sure that the rods and cones carry current of time course corresponding to the e.r.g.; the fortunate chance that the bipolar cells are the only other structures which penetrate the external limiting membrane (if we may neglect Müller's fibres) allows us to infer with fair certainty the time course of the current which their outer extremities carry; but detailed analysis of other contributions to the slow electrical activity of the frog's retina is made very uncertain by the multiplicity of probable contributors.

The above argument has been wholly in terms of carriers of current, the assumption being that the retina consists of numerous cells, with cytoplasm of low resistance and a highly resistive surface membrane, embedded in a continuous medium whose resistance is low compared with that of the surface membrane of the cells. A conclusion that the rods and cones *carry* the current responsible for the e.r.g. does not necessarily imply that they *generate* it; but the alternative assumption that they passively carry current generated by other structures is unnecessarily complex, and not very easily reconciled with the failure of separate generating structures to reveal themselves in the electrical records from eyes giving the Ottoson–Svaetichin pattern of responses. The additivity found in the e.r.g., indicating that different regions of the retina act independently of each other in generating it, gives some further support to the hypothesis that the actual generators are the rods and cones, since for them such independence is to be expected, and for elements lying centrally to such extensive tangentially orientated neurones as the horizontal cells it is not.

SUMMARY

1. A membrane of high electrical resistance and capacity (R membrane), previously described for the frog's eye, is present also in the rat and probably in the rabbit. It is absent from frogs' retinae that have been stripped from the underlying pigment epithelium. These findings are consistent with the hypothesis, earlier put forward on the basis of its distance from the anterior surface of the retina and from the sclera, that the R membrane is the external limiting membrane, to which the rods and cones act as extensions, allowing it to have the surprisingly high capacity of about $100\mu F/cm^2$.

2. Evidence is presented that the complex sequence of responses to diffuse illumination of the whole retina, commonly recorded by a micro-electrode advanced in steps through the retina of a freshly excised frog's eye, is a normal phenomenon, due neither to disturbance of the eye during dissection nor to the presence of the electrode in the region from which the recording is made.

3. The effect of intensity of stimulus on these complex intraretinal responses is very different from its effect on the e.r.g., and the complexities may be absent at all points of a retina without the e.r.g. being abnormal.

4. Different regions of the retina act independently of each other in producing the e.r.g., even when they are so near to each other that such independence is not found in the responses recorded intraretinally.

5. Results 3 and 4 are consistent with the hypothesis that the e.r.g. is generated by rods and cones. The origin of other slow electrical activity of the frog's retina is discussed.

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THE TISSUE DISTRIBUTION AND OXIDATION OF ^{14}C-LABELLED CHYLOMICRON FAT INJECTED INTRAVENOUSLY IN RATS

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Chylomicron fat labelled with glyceryl tri(1-^{14}C)palmitate is removed rapidly from the blood stream when introduced in the form of homologous chyle (Havel & Fredrickson, 1956; French & Morris, 1957). Some of the fat leaves the blood stream in the form of intact chylomicrons (Courtice & Morris, 1955; Morris & Courtice, 1956), but some may first undergo intravascular hydrolysis by a reaction analogous to the heparin clearing reaction (Robinson, Jeffries & French, 1954). The intravenous injection of heparin induces intravascular hydrolysis and increases the rate of removal of chylomicron fat from the blood stream, while protamine and Triton WR-1339 inhibit this hydrolytic reaction induced by heparin and slow down removal (Bragdon & Havel, 1954; French & Morris, 1957).

The experiments described in this paper were carried out to determine the distribution in the body of ^{14}C-labelled chylomicron fat and the extent to which it undergoes oxidation to CO_2 following its removal from the blood stream. The tissue distribution and oxidation of chylomicron fat have also been studied following the intravenous injection of heparin, protamine sulphate or Triton WR-1339 in an attempt to obtain further information on the significance of intravascular lipolysis in fat transport and metabolism.

METHODS

Albino rats of either sex were used for all experiments. The weights of the animals used for experiments on the tissue distribution of labelled chyle ranged from 170 to 190 g and for the metabolism experiments from 95 to 105 g. The rats were starved for 18 hr before use except where otherwise stated.

The method for collecting labelled chyle from rats fed with glyceryl tri[1-^{14}C]palmitate dissolved in olive oil has been described previously (French & Morris, 1957). This chyle was injected into an exposed femoral vein of the experimental animals under light ether anaesthesia. Each animal received 1 ml. of the labelled chyle which contained 40–60 mg total fatty acids. When the experiment lasted for half an hour or less, ether anaesthesia was maintained until the experiment was completed; in the metabolism experiments and in other experiments lasting longer than half an hour the animals were allowed to recover from the anaesthetic immediately after the injection.

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Heparin (Pularin, Evans Medical Supplies) was given intravenously in doses of 200 u. per rat; protamine sulphate in doses of 5 mg/kg body wt., and Triton WR-1339 (Rohm and Haas Co.) in doses of 400 mg/kg body wt. These substances were injected 1 min before the injection of the labelled chyle.

Measurement of radioactivity in tissues. At intervals after the injection of labelled chyle the animals were anaesthetized and killed by bleeding from the abdominal aorta. The liver, spleen, kidneys, lungs and a sample (1–2 g) of adipose tissue from the dorsal abdominal wall were removed from each animal and rinsed in 0·9 % saline solution. Each tissue was then ground with sand and extracted during the night with 300 ml. of a 3:1 ethanol-ether mixture. The mixture was boiled, filtered while hot and the residue re-extracted with boiling ethanol-ether and filtered. The combined filtrates were reduced by evaporation to a volume of 50 ml. The radioactivity in this extract was measured by plating 0·1 ml. portions directly on to aluminium planchets covered with lens paper and counting to an error of ±5 % with a thin mica end-window G-M tube.

The radioactivity in the chyle used in these experiments was measured by extracting 0·1 ml. with 10 ml. boiling 3:1 ethanol-ether and counting the activity in 0·1 ml. portions of this extract as described above. When the ethanol-ether extracts of tissue or chyle were re-extracted with petroleum ether the ^{4}C recoveries were not significantly changed and this step was therefore omitted from the routine procedure.

Measurements of radioactivity in the blood were made by counting samples of whole blood obtained from the tail veins and plated on to aluminium planchets as previously described (French & Morris, 1957).

Collection of expired CO_2 and measurement of its radioactivity. Immediately after the injection of labelled chyle the rat was placed in a glass metabolism cage. It was provided with water, but not food, throughout the experiment. Air free from CO_2 was supplied to the rat through an absorption line, as described by Skipper, Bryan, White & Hutchinson (1948). The expired air was bubbled through a sintered glass disk into 100 ml. of carbonate-free N sodium hydroxide to absorb the CO_2. This solution was removed for analysis and replaced by 100 ml. of fresh sodium hydroxide solution at hourly intervals for 8 hr following the injection of chyle. Absorption towers containing saturated solutions of barium hydroxide were used to check the completeness of CO_2 absorption on both sides of the metabolism chamber.

To determine the total amount and the radioactivity of the expired CO_2 in the hourly samples, the carbonate in duplicate 1 ml. portions of the solutions was precipitated by an excess of barium chloride in a glass filtration cone. The 1 ml. portions were injected into the barium chloride solution in the cone through a fine-bore hypodermic needle. This ensured a fine precipitate of barium carbonate which packed tightly during filtration. Filtration was carried out through a weighed disk (diam. 2·4 cm) of Whatman no. 1 filter paper under a stream of nitrogen. The precipitate was washed successively with CO_2-free water, 1:1 acetone–water mixture and acetone, and was dried under an infra-red lamp. The filter paper disk and precipitate were then removed from the cone and dried to a constant weight over phosphorus pentoxide. The hourly CO_2 output was calculated from the weights of the barium carbonate precipitates.

For the assay of radioactivity the samples on the filter paper were mounted in a brass holder to keep them flat and counted to an error of ±5 % with a thin mica end-window G-M tube. The radioactivity in all the barium carbonate samples was corrected to zero mass.

The radioactivity of the chyle used in these experiments was also measured as barium carbonate. Duplicate 66 µl. samples were oxidized by the procedure of Van Slyke & Folch (1940) in the apparatus described by Skipper *et al.* (1948). 5 ml. of the oxidant mixture was used and the CO_2 released was absorbed into 50 ml. of N sodium hydroxide. 5 ml. portions of this solution were then precipitated with an excess of barium chloride solution and prepared for counting as described above. The weights and measurements of radioactivity in duplicate samples agreed within ±5 %.

RESULTS

Tissue recoveries

The mean recovery of labelled fat from the various tissues at intervals after the injection of chyle intravenously is shown for groups of three rats in Table 1. The results are expressed as the percentage of the total amount of labelled fat injected which was recovered in each of the ethanol-ether extracts from the tissues.

Table 1. The recovery of labelled chylomicron fat from the tissues at intervals after the injection of 1 ml. of ^{14}C labelled chyle intravenously. The figures show the percentage of the total amount of ^{14}C injected recovered from the organs or from 1 g of adipose tissue. Mean results together with the standard errors are given for groups of three rats

	Treatment					
	Normals			Heparin		
Tissue	15 min	30 min	120 min	15 min	30 min	120 min
Blood	46·2 ±7·94	3·7 ±1·12	0·5 ±0·26	9·4 ±2·33	4·5 ±0·84	1·4 ±0·14
Liver	20·7 ±1·09	25·3 ±2·05	17·3 ±0·45	33·7 ±0·89	33·3 ±2·60	17·7 ±0·89
Spleen	3·0 ±0·90	1·6 ±0·91	0·9 ±0·06	2·1 ±0·20	1·9 ±0·84	0·3 ±0·04
Lungs	1·2 ±0·26	1·1 ±0·30	0·8 ±0·26	1·6 ±0·14	1·4 ±0·30	0·7 ±0·1
Kidneys	1·6 ±0·04	1·3 ±0·20	1·4 ±0·26	2·1 ±0·14	1·6 ±0·20	0·9 ±0
Adipose tissue 1 g	0·9 ±0·14	1·0 ±0·36	0·9 ±0·20	1·4 ±0·14	0·7 ±0·50	0·1 ±0·10

	Treatment					
	Protamine sulphate			Triton WR-1339		
Tissue	15 min	30 min	120 min	15 min	30 min	120 min
Blood	65·4 ±6·77	43·4 ±3·21	3·3 ±1·79	70·5 ±8·54	56·5 ±4·25	17·2 ±1·40
Liver	9·0 ±1·0	13·7 ±1·22	16·5 ±0·55	12·2 ±0·51	17·3 ±1·22	17·0 ±0·81
Spleen	4·8 ±0·74	5·2 ±0·41	0·5 ±0·10	4·4 ±0·84	7·2 ±1·4	2·2 ±0·28
Lungs	1·1 ±0·05	1·0 ±0·55	0·6 ±0	0·7 ±0·01	0·8 ±0·26	0·6 ±0
Kidneys	1·0 ±0·04	0·9 ±0·10	0·5 ±0·10	0·7 ±0·01	0·8 ±0·26	0·4 ±0·1
Adipose tissue 1 g	0·1 ±0·04	0·4 ±0·04	0·5 ±0·22	0·6 ±0·26	0·8 ±0·28	0·2 ±0·17

In all the experiments the liver was the organ from which the greatest amount of the injected fat was recovered. In normal animals the amount recovered reached a maximum at 30 min and then declined. Recoveries at 1, 4 and 8 hr after injection (not shown in the table) gave mean values of

22·2% ± S.E. 1·8, 15% ± 1·0 and 9·3% ± 1·8 respectively. In the treated groups there was an alteration in the time of the maximum recovery from the liver. This was obtained at 15 min in animals injected with heparin and at some time later than 30 min in animals injected with protamine or Triton. The greatest amount of labelled fat recovered from the liver at any time was obtained in the animals treated with heparin.

The recoveries from the spleen in animals treated with protamine or Triton showed higher values at 15 and 30 min than the controls. The higher recoveries at 15 min may be partly explained by retention of blood, with a relatively high level of radioactivity, in the spleen. It can be seen, however, that the recoveries obtained from the spleens of animals treated with protamine or Triton are higher at 30 than at 15 min, although the blood concentration has declined during this interval.

In all the animals only traces of labelled fat were present in the kidney and the lungs and there was no evidence that injection of heparin, protamine or Triton altered the amount of labelled fat recovered from these organs.

The recoveries from adipose tissue are expressed as the percentage of the total amount injected which was recovered per gram of adipose tissue. The results show that some labelled fat is present in the adipose tissue within 15 min following chyle injection. In view of the small amounts recovered, however, and the relatively small amount of adipose tissue analysed it is not possible to discriminate between the effects of the various treatments on the uptake by this tissue.

Recovery of ^{14}C as expired CO_2

In these experiments observations were made on animals which had received the stock diet of rat nuts (carbohydrate 56%, fat 3·9%, protein 16·9%) up to the time of the chyle injection, and on animals which had been starved for 18 hr. Fig. 1 shows the mean hourly percentages of the injected ^{14}C expired as $^{14}CO_2$ by three animals in each treatment group. The cumulative percentages of the injected ^{14}C which was expired is shown for the same experiments in Fig. 2. There was no significant difference in the total CO_2 expired between the different groups. The changes in the specific activity of the expired CO_2 with time for each group therefore showed the same relationship as that shown for ^{14}C output in Fig. 1.

In the starved animals the output of $^{14}CO_2$ followed a similar time course in the controls and in the animals injected with heparin or protamine. The maximum recovery occurred during the second hour, when means of from 15 to 20% of the injected ^{14}C appeared in the expired air as CO_2. The mean total cumulative output during the 8 hr of the experiment was approximately 60% in each of these groups. During the first 2 hr the highest hourly recoveries were obtained from the heparin-treated animals. In animals treated with

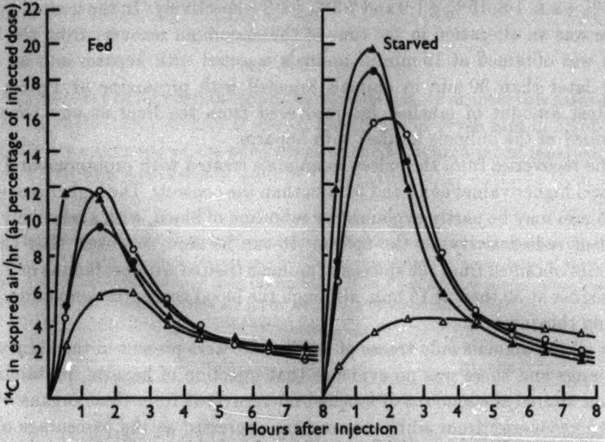

Fig. 1. The effect of intravenous injection of heparin, protamine sulphate or Triton WR-1339 on the hourly output of ^{14}C in the expired air following the intravenous injection of 1 ml. homologous chyle labelled with glyceryl tri[1-^{14}C]palmitate. Each point represents the mean value for three rats. ●—●, controls; ▲—▲, heparin 200 u.; ○—○, protamine sulphate 5 mg/kg body wt.; △—△, Triton WR-1339 400 mg/kg body wt.

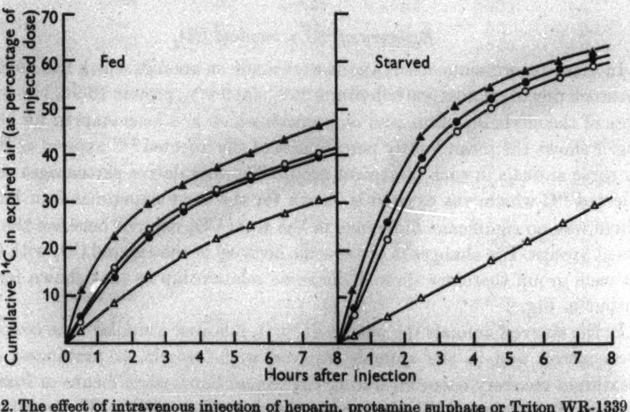

Fig. 2. The effect of intravenous injection of heparin, protamine sulphate or Triton WR-1339 on the cumulative output of ^{14}C in the expired air following the intravenous injection of 1 ml. homologous chyle labelled with glyceryl tri[1-^{14}C]palmitate. Each point represents the mean value for three rats. ●—●, controls; ▲—▲, heparin 200 u.; ○—○, protamine sulphate 5 mg/kg body wt.; △—△, Triton WR-1339 400 mg/kg body wt.

Triton there was a reduction in the percentage of the injected ^{14}C which appeared in expired CO_2; the mean maximum hourly output at 2–3 hr was 4–5% and the mean cumulative output in 8 hr was 30%.

In the animals which had been fed up to the time of the injection of chyle the percentage recovery of ^{14}C as CO_2 in normal animals and in animals treated with heparin or protamine was lower than in the corresponding groups of animals which had fasted. A mean cumulative output of 40% was obtained during 8 hr in the control and protamine-treated groups. The mean cumulative output in animals treated with heparin was 47%. Animals treated with Triton again showed the lowest output of $^{14}CO_2$ and expired a mean of about 30% in 8 hr. The maximum hourly production of $^{14}CO_2$ occurred in the first hour in the animals injected with heparin, in the second hour in the controls and animals injected with protamine, and in the third hour in animals injected with Triton.

DISCUSSION

The role of various organs in the removal of emulsified fat from the blood and the rate of oxidation in the tissues has been studied extensively by the use of artificial emulsions given intravenously (Lerner, Chaikoff, Entenman & Dauben, 1949; Rutenberg, Seligman & Fine, 1949; Goldman, Chaikoff, Reinhardt, Entenman & Dauben, 1950; Geyer, Waddell, Pendergast & Yee, 1951; Waddell, Geyer, Clarke & Stare, 1953; Becker, Rall & Grossman, 1955). These findings cannot, however, be related directly to the behaviour of chylomicron fat since it has been found that the results are altered by such factors as the stability of the emulsion, the nature of the emulsifying agent and the size of the emulsified particles.

Studies in which homologous chyle has been used have been restricted to observations on the tissue distribution of the injected fat. Markowitz & Mann (1930) showed that, in contrast to the findings with many artificial emulsions, there was no increase in fat in the lungs following intravenous injection of citrated chyle. Murray & Freeman (1951) compared by histological methods the distribution of chyle fat and an artificial fat emulsion in the tissues of rats and dogs. They found that the artificial emulsion was concentrated in the spleen, the lungs and the Kupffer cells of the liver after intravenous injection. After intravenous injection of chyle, on the other hand, the increase in stainable fat was found mainly in the parenchymal cells of the liver. Morris & Courtice (1956) demonstrated accumulation of fat in the parenchymal cells of the cat's liver after intravenous infusion of large volumes of homologous chyle.

The present investigations confirm the finding that the liver is a major site of uptake for chylomicron fat and that negligible quantities are removed by the kidneys, lungs and spleen. The recoveries from adipose tissue, although indicating that a proportion of the chylomicron fat appeared in the fat depots within the experimental period, cannot be interpreted quantitatively. It is not known whether the sample taken was representative of the adipose tissue as a whole or what was the total amount of depot fat in the different animals. As a crude estimate, assuming that 7–10% of the weight of the animals used consisted of depot fat (cf. Reed, Yamaguchi, Anderson & Mendel, 1930), it appears that the fat depots contained amounts of the order of 10% of the labelled fat at each interval in the normal animals. Lerner *et al.* (1949) recovered 6–8% of a labelled tripalmitin emulsion from depot fat in rats 24 hr after injecting the emulsion intravenously.

The total recoveries at the various intervals, when allowance was made for the total fat depots and for the amount oxidized, still failed to account for the total amount of labelled fat which had disappeared from the blood. In addition to the uptake by the various tissues analysed, which accounted for about 15% of the total body weight of the animal, there was some general uptake of labelled fat by tissues throughout the carcass. Some of the labelled fat distributed in the carcass and fat depots may have represented fat which was taken up initially by the liver and then retransported, since it is known that an exchange of fatty acids between the liver and other tissues occurs via the blood (Harper, Neal & Hlavacek, 1953). The present experiments show, however, that chylomicron fat is taken up directly by extrahepatic tissues. The rate at which the amount of labelled fat in the liver decreased during the 8 hr of the experiment indicated that the exchange of fatty acids from the liver was insufficient to account for the initial rapid uptake by extrahepatic tissues. Using artificial emulsions, the uptake and utilization of labelled tripalmitin by extrahepatic tissues has been demonstrated conclusively in animals from which the liver has been removed (Goldman *et al.* 1950; Geyer *et al.* 1951).

The experiments have shown that the triglycerides present in homologous chylomicrons are readily available for oxidation by the tissues. Comparisons with the other available data suggest that when tripalmitin enters the blood in this form it is oxidized more readily than when given as an artificially stabilized emulsion. The rates of oxidation of labelled chylomicron fat are influenced, however, by the dietary state of the animal and by the amount of chyle injected. The results of experiments under these varying conditions in the rat are reported more fully elsewhere (Morris, 1958).

The increased rate of removal of chylomicron fat from the blood which follows the injection of heparin is associated with an increase in its rate of uptake by the liver. Heparin injection also increases the relative amount of the injected fat going to the liver. The values obtained, however, indicate that increased liver uptake does not by itself account for the increased rate of removal from the blood, and it can therefore be inferred that the uptake of chylomicron fat at other sites in the carcass is also directly accelerated by heparin.

Heparin increases the amount of chylomicron fat oxidized to CO_2 in the first 1–2 hr following the chyle injection. This effect is seen most clearly in the fed group of animals, in which the output of $^{14}CO_2$ is much higher in the heparin-treated animals in the first hour. These results show that following heparin injection the injected fat becomes more rapidly available for oxidation. There is no evidence from the present experiments that heparin affects the total CO_2 output or respiratory quotient, as might be expected if it had a more specific effect in fat metabolism.

Since the effects of heparin are believed to be due to intravascular hydrolysis of chylomicron fat (cf. Robinson & French, 1957) it appears that the tissues are able to take up unesterified fatty acids more rapidly than intact chylomicrons. This is supported by observations by Havel & Fredrickson (1956), which showed a very rapid removal of labelled fat from the blood when it was introduced in the form of unesterified fatty acid. In addition, the fat entering the cells in this form is presumably more readily oxidized to CO_2 since the essential step of hydrolysis of triglyceride has already occurred. These differences in the uptake and rate of oxidation of hydrolysed and unhydrolysed chylomicron fat have recently been demonstrated using the isolated perfused rat liver (Morris & French, 1958). It was found that labelled fat was taken up and oxidized more rapidly by the liver when the perfusate contained prehydrolysed fat than when it contained intact chylomicrons.

The effect of protamine and Triton WR-1339 in delaying the uptake of chylomicron fat by the liver may possibly be due to inhibition of intravascular lipolysis since these substances are known to inhibit the clearing reaction *in vitro* (Robinson *et al.* 1954). On the other hand, the increased uptake by the spleen suggests that these substances have an additional effect *in vivo*. Triton WR-1339 is a surface-active agent and protamine in suitable concentrations causes clumping and flocculation of chylomicrons (Brown, 1953). It seems probable, therefore, that an alteration in the surface properties of the chylomicrons may lead to their uptake by reticulo-endothelial cells in the spleen. Under these conditions the distribution of chylomicron fat resembles more closely that observed when artificial emulsions are injected into the circulation. In addition, Triton WR-1339 has an effect on the oxidation of chylomicron fat which cannot be explained entirely by the delayed tissue uptake. The delayed uptake of chylomicron fat which occurred in the presence of protamine was not associated with any significant reduction in the amount of fat oxidized during the first 1–2 hr. On the other hand, although the rate of uptake by the tissues was reduced to a similar extent by Triton WR-1339, this substance caused a marked reduction in the rate at which the fat was oxidized.

SUMMARY

1. Chylomicron fat, labelled with glyceryl tri(1-^{14}C)palmitate, is taken up from the blood stream principally by the liver but also by other tissues. It does not accumulate in the lungs or spleen.

2. The labelled fat is rapidly oxidized. Following the injection of 1 ml. labelled chyle into fasting rats, the $^{14}CO_2$ in the expired air reaches a maximum concentration in 2 hr and accounts for about 60 % of the injected fat after 8 hr.

3. The injection of heparin accelerates the uptake of chylomicron fat, particularly by the liver. Following heparin injection the fat is also more rapidly available for oxidation.

4. Inhibitors of the heparin clearing reaction, protamine sulphate and Triton WR-1339, delay the uptake of the labelled fat by the liver but do not prevent it. These substances cause an accumulation of the fat in the spleen.

5. Protamine sulphate in doses which inhibit the heparin clearing reaction in the blood has no detectable effect on the proportion of chylomicron fat oxidized in 2 hr. In the presence of Triton WR-1339 chylomicron fat is oxidized more slowly.

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THE EFFECT OF CALCIUM AND pH ON THE ANAPHYLACTIC REACTION

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In two previous papers (Mongar & Schild, 1957*a*, *b*) we have studied the effects of inhibitors of anaphylaxis and formulated a scheme for the anaphylactic reaction based on the action of these inhibitors. The present paper is a continuation of this work, and deals mainly with the effects of calcium and pH on the anaphylactic reaction.

Several immunological reactions are known to require calcium. Levine, Cowan, Osler & Mayer (1953*a*, *b*) have shown that calcium is required for immune haemolysis and that it is concerned with the binding of complement to the antigen-antibody complex. Humphrey & Jacques (1955) found that calcium lack inhibits the release of 5-hydroxytryptamine from platelets, which occurs during the antigen–antibody reaction in rabbit serum. The release of histamine by antigen from the formed elements of rabbit's blood is inhibited by oxalate and citrate but high concentrations of calcium (20 mM) also produce inhibition (McIntire, Roth & Richards, 1949). Citrate also inhibits certain allergic skin reactions (Rocha e Silva, 1952). The present work shows that the release of histamine from sensitized guinea-pig lung in anaphylaxis is inhibited by calcium lack and by a reduction in pH and that the effects of calcium and pH on the anaphylactic reaction are interdependent. A brief report of these findings has already been published (Mongar & Schild, 1957*c*).

METHODS

Guinea-pigs were actively sensitized with egg albumin and samples of chopped lung were prepared by the method previously described (Mongar & Schild, 1956) with the following minor modifications: For the study of the effects of ions the chopped lung was washed in isotonic sucrose instead of Tyrode solution. For the study of the effect of pH, phosphate or bicarbonate buffer was used, and in each case the final pH of the solution after incubation with the tissue was determined electrometrically. One millilitre of isotonic phosphate buffer was added to 5 ml. of Tyrode solution. When bicarbonate buffer was used the pH was varied either by altering the concentration of $NaHCO_3$ at constant CO_2 concentration, or by altering the concentration of CO_2 at constant $NaHCO_3$ concentration. Different mixtures of CO_2 and O_2 were provided in cylinders by the British Oxygen Company. The gas was bubbled into the solution, in a beaker fitted with a rubber cap, through a hypodermic needle, which was raised above the level of the liquid after addition of the antigen (10^{-3} egg albumin) to avoid frothing. The histamine content of solutions was measured on the atropinized guinea-pig ileum and is expressed in terms of the content of histamine in the lung particles, which ranged between 7 and 20 μg of histamine base per gram of tissue.

RESULTS

The effect of calcium

When sensitized guinea-pig lung is placed in Tyrode solution containing antigen, about 20–50 % of the tissue histamine is released into the surrounding fluid. When Tyrode solution from which calcium chloride has been omitted is used instead, histamine release with antigen is greatly diminished, and when Tyrode solution is used to which a calcium chelating agent (0·05 % versene; ethylenediaminetetraacetate) has been added, histamine release with antigen is reduced to the control level in the absence of antigen (Fig. 1).

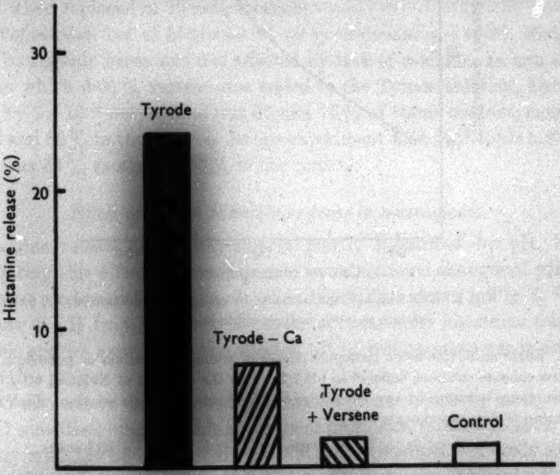


Fig. 1. Effect of removal of calcium from Tyrode solution on histamine release by antigen from chopped sensitized guinea-pig lung. Release is expressed as a percentage of the tissue histamine. The columns show partial inhibition of histamine release when $CaCl_2$ is omitted from the Tyrode solution and complete inhibition when 0·05 % versene is added to the Tyrode solution. In each case the lung was left for a period of 15 min in the modified Tyrode solution followed by a further 15 min period in the presence of antigen.

When sensitized lung is placed in saline solution (0·9 % NaCl) containing antigen, very little histamine release occurs, but the anaphylactic mechanism recovers if calcium is added and the degree of recovery depends on the concentration of calcium, as is shown in Fig. 2. Even with 10 mM calcium the release is not completely restored to the level in Tyrode solution. This also requires the addition of buffer. Magnesium and potassium ions are, however, not required, as will be shown in the next section. The omission of calcium ions from the solution, without addition of a calcium chelating agent, is usually insufficient to abolish histamine release completely. In four experiments in saline the mean release of histamine with antigen was 8 ± 2 % of the Tyrode control, whereas in four experiments in saline containing 0·01 % versene, it was only 3 ± 1 % of the Tyrode control, which is not significantly different from the spontaneous release which occurs in the absence of antigen.

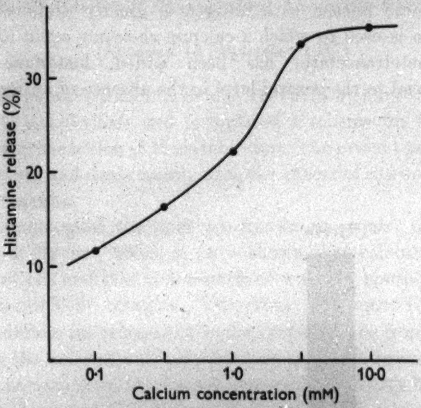


Fig. 2. Effect on histamine release by antigen of addition of calcium to 0·9 % sodium chloride solution.

TABLE 1. Effect on anaphylactic histamine release of adding magnesium or potassium ions to sodium chloride solutions buffered to pH 7·2–7·3 by the addition of $NaHCO_3$ 0·1 g/l. Histamine release is expressed as a percentage of the release in Tyrode solution. Each result is the mean of four experiments

	MgCl$_2$ concn. (g/l.)			KCl concn. (g/l.)		
	0	0·1	1·0	0	0·2	2·0
Histamine release (%)	7·4	4·0	3·5	7·4	6·3	2·9

Effect of magnesium and potassium. When magnesium or potassium ions were omitted from Tyrode solution, histamine release was in no way affected. In three experiments in which histamine release in Tyrode solution was compared with release in Tyrode solution from which magnesium and potassium ions were omitted, the following results were obtained: histamine release in Tyrode solution, 23, 40 and 47 %; histamine release in Mg- and K-free Tyrode solution, 25, 36 and 46 %. When magnesium or potassium ions were added to saline they failed to restore the anaphylactic mechanism. Table 1 shows that the addition of these ions to a buffered solution of sodium chloride, in concentrations equal to or ten times greater than those found in Tyrode solution, does not increase histamine release above that in saline, but if anything depresses it further.

Effect of sucrose. When sodium chloride was replaced by sucrose, histamine release was reduced but not abolished provided that calcium was also present. Sensitized lung suspended in buffered isotonic sucrose (with $KHCO_3$, 0·05 g/l.) released no histamine with antigen, but when 1 mM calcium was added to the sucrose solution histamine release by antigen was 32 %, and with 10 mM 42 %, of the Tyrode control (mean of four experiments). Although the anaphylactic mechanism does not function in pure sucrose solution it is not permanently damaged even by prolonged immersion in sucrose. It was found that sensitized lung tissue which was left in sucrose for up to 3 hr reacted normally with antigen when replaced in Tyrode solution.

Effect of calcium lack on histamine release by octylamine and 48/80. Histamine release by organic bases was not affected by lack of calcium. In two experiments in which 0·05 % versene was added to the Tyrode solution, histamine release by 2×10^{-4} octylamine was 53 and 76 % of tissue content, compared with 62 and 56 % in the control. In one experiment with 10^{-3} 48/80 histamine release was 33 % as against 35 % in the control.

Effect of pH on histamine release in anaphylaxis

Histamine release in anaphylaxis is greatly influenced by pH, but in investigating this effect it is necessary to avoid extreme changes of pH since these cause a release of histamine by themselves, as is shown in Fig. 3. Within the range of pH from 6 to 8·5, however, 'spontaneous' histamine release is negligible and we have tested the effect of pH on histamine release in anaphylaxis in this range.

Fig. 3 also shows the effect of pH on histamine release by antigen from chopped sensitized lung. In this experiment phosphate buffer solutions, where necessary with the addition of HCl or NaOH, were added to Tyrode solution to give pH values ranging from 5·3 to 9·1. In each case the final pH of the solution after incubation with the tissue particles was checked electrometrically. The curve for histamine release by antigen has a maximum at pH 7·8 and a minimum at pH 6·2–6·5. Details of five experiments are given in Table 2. In each the release curve exhibits a minimum in the range pH 6–6·5, and a maximum in the range pH 7·6–7·9. The mean value for the maximum is at 7·75, and for the minimum (in the two best experiments) at 6·3. A change of pH from 6·3 to 7·8 caused a 25-fold increase in histamine release. Above pH 8 the curve shows a distinct downward trend, but this section of the curve could not be explored further in view of the large spontaneous histamine release

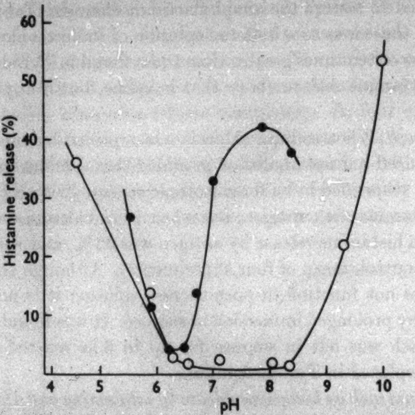


Fig. 3. Effect of pH on histamine release in anaphylaxis (phosphate buffer): ●, release from sensitized lung by antigen; ○, release in absence of antigen.

Table 2. Effect of pH on histamine release by antigen (egg albumin 1 mg/ml.). Each experiment was done on samples of chopped lung from a different guinea-pig

Expt. 1		Expt. 2		Expt. 3		Expt. 4		Expt. 5	
pH	Release (%)	pH	Release (%)	pH	Release (%)	pH	Release (%)	pH	Release (%)
6·2	37	5·3	18	6·0	19	5·55	14	5·52	27
7·1	54	6·0	10	6·1	20	6·1	2·5	5·83	12
7·65	57	7·5	29	7·65	72	6·3	3	6·22	4
8·0	52	8·3	27	8·4	58	7·4	43	6·48	5
8·25	47	9·0	54	9·1	58	8·3	48	6·68	14
						8·6	30	7·0	33
								7·9	42
								8·38	37

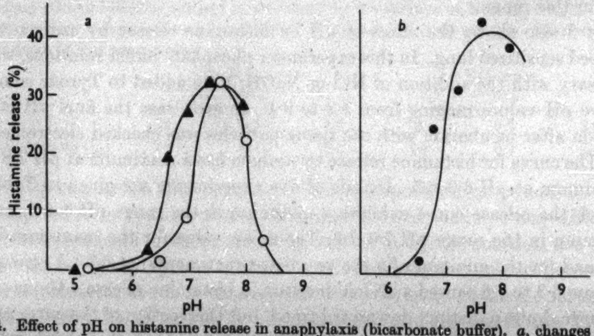


Fig. 4. Effect of pH on histamine release in anaphylaxis (bicarbonate buffer). *a*, changes of pH produced by varying the concentration of bicarbonate; *b*, proportion of CO_2 in O_2–CO_2 mixture varied.

occurring above pH 8·5. Nevertheless, there could be no doubt about the significance of the effect; at pH 8·4 histamine release was reduced on the average to 83% of the maximum.

Two further series of experiments, in which bicarbonate instead of phosphate buffer was used, are illustrated in Fig. 4. In one of these the bicarbonate concentration was varied from 0 to 10 g/l. and the buffering produced by aerating the solutions with oxygen containing 5% CO_2 (Fig. 4*a*). In the other series (Fig. 4*b*) the concentration of bicarbonate was maintained constant at 0·1 g/l. and the pH was varied by bubbling oxygen containing varying amounts of carbon dioxide (0·03–100%). The curves obtained with bicarbonate buffer are similar to those with phosphate buffer in having a maximum at pH 7·5–7·6 and a minimum at pH 6·5, but they differ in the lowest pH range. In phosphate buffer, histamine release at pH 5·5 was definitely greater than at pH 6, whereas in bicarbonate buffer the curve shows no upward trend at values below pH 6. The release of histamine in the presence of antigen by phosphate Tyrode solution below pH 6 can be attributed to tissue damage, since a similar release was obtained in the absence of antigen. In Tyrode solution of pH 5·2, produced by bubbling carbon dioxide without bicarbonate, no histamine release occurred either in the presence or in the absence of antigen.

Interaction of calcium and pH

The effects of calcium and pH on histamine release in anaphylaxis are closely connected. Calcium lack can be compensated for by increasing the pH. Fig. 5 shows the effect of pH in solutions containing saline with and without versene (*a* and *b*) and saline with calcium (*c*). In the complete absence of calcium (0·05% versene) there is no histamine release which could be attributed to antigen, the curve coinciding with the curve of spontaneous release. In calcium-free solutions, but without versene, there is no histamine release at pH 7 but an appreciable release at pH 8. In solutions containing 1 mM calcium there is reduced histamine release at pH 7 but a strong release, comparable to that in Tyrode solution, at pH 7·8.

Inhibition in a solution of low pH can be compensated for by increasing the concentration of calcium. Fig. 6 shows the effects of calcium on histamine release by antigen at pH 6 and 8. The calcium concentration in the Tyrode solution was varied from 1 to 40 mM. At pH 8 there was no effect of calcium concentration on histamine release. At pH 6 histamine release was greatly reduced in 1 mM calcium, but in 5–40 mM calcium it was almost equal to the control value at pH 8.

Effect of calcium lack and acidity on desensitization

It was shown in earlier work that inhibition of histamine release in anaphylaxis may be produced without interference with desensitization. Thus phenol

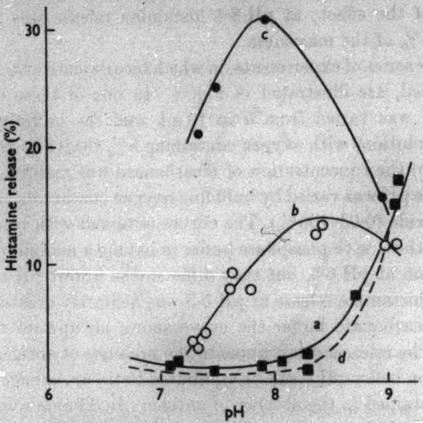


Fig. 5. Curves *a*, *b* and *c* represent histamine release by antigen from sensitized lung: *a*, saline with 0·05% versene; *b*, saline alone; *c*, saline with 1 mM Ca. Curve *d*, histamine release without antigen.

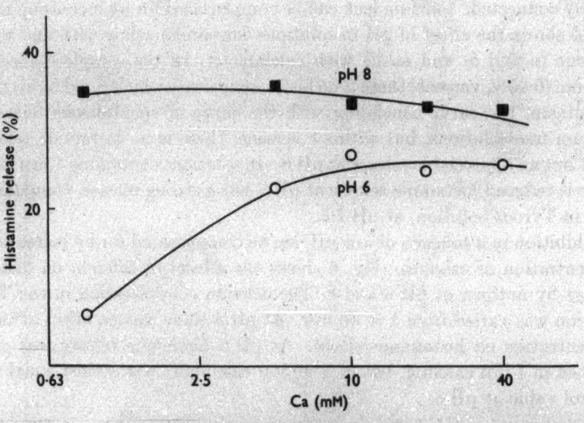


Fig. 6. Effect of calcium concentration with change in pH. Each point represents the mean of two experiments. In the experiments at pH 6 the bicarbonate of the Tyrode solution was omitted and CO_2 5%–O_2 95% was bubbled through the solution. The tonicity of the solutions with high calcium was adjusted by reducing the NaCl correspondingly.

blocks histamine release by antigen from sensitized tissue (Mongar & Schild, 1957*a*), but after removal of the phenol the tissue is completely desensitized. On the other hand, reduction of temperature also blocks histamine release by antigen, but when the tissue is warmed up again it is found to be incompletely desensitized as is shown by the fact that it now releases histamine (Mongar & Schild, 1957*b*). The following experiments show the effect of calcium lack on desensitization.

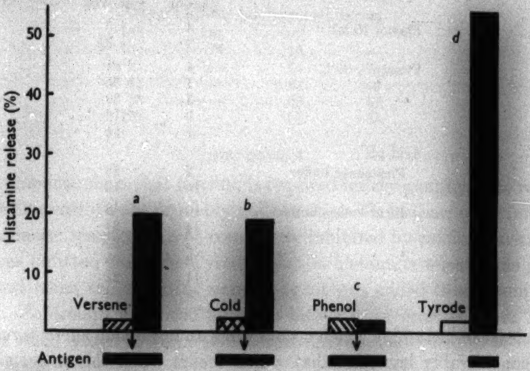


Fig. 7. Histamine release after removal of the inhibitor. In Expts. *a*, *b* and *c* the antigen was present throughout but the inhibitor was removed half way through the experiment. Expt. *d* was a control showing, first, spontaneous release and then histamine release by antigen in the absence of inhibitor.

Antigen was applied to sensitized lung suspended in calcium-free Tyrode solution containing 0·05% versene and, as expected, it failed to produce a release of histamine. After 15 min the lung was placed in ordinary Tyrode solution containing antigen and it then released a substantial quantity of histamine (30% of control). This experiment is illustrated in Fig. 7*a*, along with corresponding experiments with low temperature (7*b*) and phenol (7*c*). After removal of the block by low temperature a release of histamine occurs of the same magnitude as after removal of the block by versene, whereas after removal of the phenol block no residual histamine release occurs. It is thus clear that calcium lack, like low temperature but unlike phenol, causes a partial inhibition of desensitization which results in a release of histamine when the calcium is replaced.

Table 3 shows the effects of phenol, versene and acid pH, and their subsequent replacement by normal Tyrode solution, on histamine release by antigen. Lack of calcium combined with 0·05% versene produced complete inhibition, and 10 mM phenol and a pH of 6·2 caused substantial reduction of histamine release. Removal of block by versene was always followed by histamine release; removal of block by acid pH was also followed by histamine release, though not as consistently as after versene; but removal of block by phenol was not followed by histamine release.

TABLE 3. Histamine release by antigen in the presence and after removal of various inhibitors. The two columns represent releases in successive 15 min periods, expressed as percentage of control and corrected for spontaneous release

	Inhibitor present	Inhibitor removed
Phenol 10 mM	4	5
	7	3½
Versene 0·05%	4	81
	−1	35
	−3	39
	0	16
	−1	14
Acid pH		
Phosphate buffer	2	48
	16	13
Bicarbonate buffer	10	48
	14	31
	2	37
	6	28
	11	22
	5	4

The release of histamine which occurs when an inhibiting agent is removed is always considerably less than that which occurs when the antigen is added without inhibitor. This difference can be interpreted in two ways: it might indicate a partial desensitization or it might be due to persistent damage by the inhibitor. The following experiments show, however, that none of the inhibitors used produced a persistent damage. After 15 min exposure of sensitized lung to a solution containing 0·05% versene (but no antigen) a subsequent anaphylactic reaction in Tyrode gave a release of histamine which was 120% of control; similarly, after exposure to a solution of pH 6 (without antigen) the subsequent histamine release was 110% of control and after exposure to a solution containing 10 mM phenol it was 90% of control. It thus seems that neither calcium-lack nor a reduction of pH to 6·2 damages the tissue, and that both induce a partial desensitization.

Effect of Ca lack and acid pH on oxygen consumption

All inhibitors of anaphylaxis previously studied inhibited oxygen consumption as well as histamine release. Calcium lack differs from these in producing a complete inhibition of histamine release in anaphylaxis without inhibiting oxygen consumption.

Oxygen uptake was measured by the manometric method. Chopped lung was suspended in calcium-free Tyrode solution in which the bicarbonate was replaced by phosphate buffer at pH 7·6 with and without 0·05% versene.

Similar experiments were also carried out in Tyrode containing phosphate buffer at pH 6·2. The results of these experiments are shown in Table 4. Neither calcium lack nor a pH of 6·2 produced an appreciable diminution of the oxygen consumption of chopped guinea-pig lung.

TABLE 4. Effect of calcium lack and acid pH on oxygen uptake of chopped lung tissue

Control	Ca-free	Versene (5×10^{-4})	Acid pH
Oxygen uptake (μl./hr/0·2 ml. tissue)			
185	184	197	—
100	78	76	—
83	—	94	71
41	—	34	43
58	—	63	42
102	—	97	85

DISCUSSION

These experiments show that calcium is required for the anaphylactic reaction and that other cations are probably not essential. The release of histamine in the anaphylactic reaction can be completely inhibited by calcium lack. When calcium was omitted from the Tyrode solution histamine release was greatly reduced and when the chelating versene agent was added it was completely abolished. Potassium produced no effect either when omitted from Tyrode solution or when added to saline solution; it thus does not seem to be required in anaphylaxis.

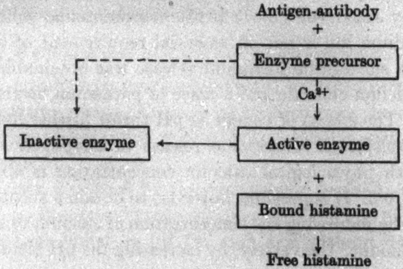
Magnesium also produced no effect when it was omitted from Tyrode solution or added to saline. But our experiments do not exclude the possibility that a trace of this ion may nevertheless be required, since complete abolition of histamine release was only obtained by the addition of versene which binds both calcium and magnesium. On the other hand, the negative results obtained by adding magnesium to saline show that magnesium cannot replace calcium. There was some evidence that magnesium, rather than augmenting the effects of calcium, slightly antagonized these, but this antagonism did not increase with the concentration of magnesium and there was no suggestion of a competitive antagonism between the two divalent cations such as was found by Jenkinson (1957) at the neuromuscular junction.

Even the sodium ion is not an essential requirement of the anaphylactic reaction since a substantial histamine release was obtainable in sucrose containing only calcium chloride and a trace of potassium bicarbonate.

Effect of pH. The effects of change in pH throw further light on the role of calcium in anaphylaxis. Histamine release in anaphylaxis is maximal at pH 7·8 and with physiological calcium concentration is almost completely inhibited at pH 6·2. It is possible, however, to obtain a substantial histamine release at pH 6 by increasing the concentration of calcium to several times the physiological amount. Conversely, by increasing the pH histamine release can be obtained in a solution containing no free calcium ions. The effects of calcium and pH are thus interdependent; in order to preserve the anaphylactic reaction at reduced pH the concentration of ionized calcium must be increased. A possible interpretation of these findings is that the anaphylactic reaction requires bound calcium and that the binding decreases with pH. It is unlikely that calcium is bound to the carboxyl groups of protein, as it is in the Ca-casein complex, for this binding is unaffected by pH in the range 6·3–8·5 (Chanutin, Ludewig & Masket, 1942). It is possible, however, that Ca combines with the imidazole groups of a protein, since the nitrogen atom of the imidazole ring can form complexes with divalent cations (Gurd & Wilcox, 1956) which dissociate at about pH 6 (Tanford, 1952).

Mode of action of calcium. Low calcium is known to increase the stability of mitochondria, and the inhibition of histamine release in anaphylaxis might conceivably be due to increase of stability of the mitochondrial particles which contain histamine. This seems unlikely, however, since the action of other histamine releasers such as octylamine and compound 48/80 is unaffected by calcium lack. Another possibility to be considered is that calcium may be required for the union of antigen with antibody. There is, however, no evidence for such an action of calcium from the *in vitro* studies of Levine *et al.* (1953*a*), who showed that the antigen-antibody reaction takes place equally in the presence and in the absence of calcium.

A more probable mode of action of calcium is based on an analogy with complement fixation which, as shown by Levine *et al.* (1953*b*), requires the presence of calcium. In a previous communication (Mongar & Schild, 1957*b*), we have suggested a scheme of the anaphylactic reaction which involves a cellular mechanism which at one stage resembles the complement-fixation reaction. It was suggested that the antigen-antibody complex combines with a heat-labile protein and that this leads to the activation of an unstable enzyme system which is concerned in the process of histamine release. Calcium may be required in this activation in much the same way as it is for the activation of the first component of complement (Levine & Mayer, 1954). This possible role of calcium is indicated below:



It is supported by the finding that when antigen is added to sensitized lung in the absence of calcium, and calcium is added afterwards, histamine release only occurs when the calcium is added. Nevertheless, the histamine release which occurs under these conditions is not as great as the normal and some explanation is required for an apparent partial desensitization in the absence of calcium.

It may, perhaps, be postulated that in the absence of calcium the enzyme precursor is partly converted to an inactive product (indicated by the broken line). An analogy to this is provided by the transformation of trypsinogen, which in the presence of calcium is converted (by the autocatalytic effect of a trace of trypsin) into trypsin but in the absence of calcium is mainly converted into inactive products (McDonald & Kunitz, 1941).

Oxygen consumption. In previous work on inhibition of anaphylaxis (Mongar & Schild, 1957*a*), it was found that all substances which inhibit histamine release in anaphylaxis also inhibited oxygen consumption. Some of the substances tested such as dinitrophenol inhibited oxygen consumption much more than histamine release, others such as antipyretics inhibited both processes in about the same concentrations, but none inhibited anaphylaxis without depressing oxygen consumption. These findings, coupled with the fact that anoxia also inhibits histamine release in anaphylaxis, seemed to throw doubt on the possibility of discovering a selective inhibitor of anaphylaxis, but the fact that calcium lack and acid pH inhibit anaphylaxis but not oxygen consumption shows that it is possible to block the anaphylactic reaction without affecting the cell as a whole.

SUMMARY

1. Histamine release in anaphylaxis from chopped, actively sensitized guinea-pig lung is greatly reduced if calcium ions are omitted from the Tyrode solution in which the lung particles are suspended, and is abolished if 0·01 % versene (ethylenediaminetetraacetate) is added.

2. Histamine release by compound 48/80 and octylamine is not reduced by calcium lack.

3. Omission of potassium and magnesium ions does not affect the anaphylactic histamine release and replacement of sodium ions by sucrose reduces but does not abolish it. Addition of calcium ions to a buffered sodium chloride solution restores the anaphylactic histamine-releasing mechanism whereas the addition of magnesium or potassium ions is ineffective. It thus seems that the anaphylactic reaction requires the presence of calcium ions but not magnesium, potassium or even sodium ions.

4. Histamine release in anaphylaxis is pH-dependent. It has an optimum at about 7·8 and falls off at higher and lower values. At pH 6·2 it is almost abolished.

5. The effects of calcium and pH are interdependent. Inhibition by low pH can be counteracted by adding calcium to the solution and inhibition by low calcium can be counteracted by making the solution more alkaline. These findings suggest the formation of a calcium-protein complex which dissociates at an acid pH.

6. Calcium lack not only inhibits histamine release in anaphylaxis but also interferes with desensitization. It is concluded that calcium is probably needed in the activation of an enzyme system required in the anaphylactic reaction.

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FORMATION OF PLASMA KININS BY PLASMIN

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Vasodilator polypeptides appear to play some part in the physiology and pathology of vascular control, so that the determination of the conditions under which they occur in the circulation, and an understanding of the mechanism of their formation, are required for a general understanding of the control of peripheral blood vessels. The present experiments describe the formation by plasmin of vasodilator polypeptides, plasma kinins, which is a name applied to bradykinin-like polypeptides derived from plasma proteins. The term plasma kinin and its definition was suggested in a discussion between J. H. Gaddum, S. M. Hilton, C. A. Keele, M. Schachter and myself at the meeting of the British Pharmacological Society at Oxford in June 1957. Our present knowledge about vasodilator polypeptides stems mainly from two groups of workers, Frey, Kraut and Werle in Germany, and Rocha e Silva and Beraldo and their colleagues in Brazil. Frey, Kraut & Werle (1950), isolated, in a crude form, from several tissues and body fluids, a material which caused a fall in blood pressure and had some of the characteristics of an enzyme. Although it is not certain if each of these materials contain the same active principle they have all been given the name of kallikrein. In 1937 Werle, Götze & Keppler made the observation that when kallikrein came into contact with blood proteins a second substance [illegible] ("kallidin") was formed, which was not a protein like kallikrein but a smaller molecule. However, it was not fully appreciated that this second substance was the agent principally responsible for the actions of kallikrein until Rocha e Silva, Beraldo & Rosenfeld (1949) observed that trypsin and the proteolytic enzymes in certain snake venoms, on incubation with plasma proteins, formed a polypeptide which they called bradykinin and which was later found to be indistinguishable from the second substance of the German workers, called kallidin.

It seems likely that there are several proteolytic enzymes capable of splitting one of the plasma proteins, probably an α_2 globulin, in a similar manner, producing a group of closely related polypeptides with powerful smooth muscle stimulating and vasodilator activity. Substances of this group are referred to as plasma kinins. It seems probable that the plasma kinins, bradykinin and kallidin are in fact mixtures of polypeptides and when using these terms on the following pages this possibility is not meant to be excluded.

Plasmin (fibrinolysin) is a proteolytic enzyme which is found in blood, normally present as an inactive precursor called plasminogen (Christensen & MacLeod, 1945). Recently, Scandinavian workers (Astrup, 1956; Mullertz, 1956) have discovered that extracts of many tissues are capable of activating plasminogen to the active proteolytic enzyme. If plasmin were able to form vasodilator plasma kinins, injured tissues, containing plasminogen activators, would form vasodilator polypeptides and these would then take part in the tissue reaction. Evidence in favour of the view that a smooth muscle stimulating substance is formed by plasmin has already been reported by several authors. Beraldo (1950) and Schachter (1956) have shown that bovine plasmin gives rise to smooth muscle stimulating activity when incubated with plasma proteins and Lewis & Work (1957), as well as Armstrong, Jepson, Keele & Stewart (1957), found the same for human plasmin. In the present experiments it will be shown that the plasma kinin formed by human plasmin is indistinguishable from bradykinin. Further, using proteolytic inhibitors it was found that the activation of plasma when treated with chloroform or when brought into contact with a glass surface results from the activation of plasminogen to plasmin with the subsequent formation of a plasma kinin.

METHODS

In order to test whether plasmin acted upon plasma proteins to form a smooth muscle stimulating substance, samples of the enzyme preparations were incubated with pseudo-globulin solution and the incubated mixture tested on isolated smooth muscle preparations.

The principle in the incubated mixture of plasmin and pseudo-globulin solution responsible for smooth muscle stimulating activity was then extracted, using the same procedure as that employed by Andrade & Rocha e Silva (1957) for the extraction of crude bradykinin from an incubated mixture of snake venom and pseudo-globulin. For this purpose human plasmin 1 g (Merck, Sharpe & Dohme) was incubated with 50 ml. pseudo-globulin solution at 37° C for 20 min. Two volumes of boiling absolute alcohol were added and the mixture boiled for a further 10 min. After centrifugation, the filtrate was evaporated to dryness *in vacuo* at a temperature below 50° C. The residue was taken up in glacial acetic acid and precipitated with 9 volumes of ether. The powder prepared in this way was compared with a standard preparation of bradykinin on the following preparations.

Smooth muscle preparations. The isolated guinea-pig's ileum, rat's uterus and rat's colon were suspended in a 15 ml. organ bath. For the guinea-pig's ileum the bath fluid was Mg-free Tyrode solution containing atropine (10^{-8} g/l.), maintained at 34° C; and for the rat's tissues de Jalon's solution was used at 28° C for the uterus and at 24° C for the colon.

Vasodilatation was examined in the skin of the hind leg supplied by the saphenous artery. Cats were anaesthetized with pentobarbitone sodium (40 mg/kg) and the femoral artery dissected beyond the origin of the saphenous artery, all muscle branches being tied off. A cannula for arterial injection was then inserted into the femoral artery beyond the origin of the saphenous artery. Since all branches of the femoral artery below the inguinal ligament were ligated, substances in a volume of 0·2–0·3 ml. would be carried by the blood stream into the saphenous artery. The femoral vein was also dissected and its side branches except the saphenous vein were ligated. The femoral vein was then tied distal to the saphenous vein and a cannula inserted into the femoral vein so as to allow the blood from the saphenous vein to pass a photo-electric drop counter after the cat had been given heparin (10 mg/kg). The outflow was recorded with a Gaddum drop timer, and the blood allowed to pass back into the femoral vein.

Estimation of plasmin. The proteolytic activity of the enzyme preparations was determined by (*a*) hydrolysis of casein, measuring the increase in optical density at 275 mμ of the deproteinized reaction mixture as described by Mullertz (1955), (*b*) hydrolysis of benzoylarginine ethyl ester, measuring optical density at 253 mμ, employing the method of Schwert & Takenake (1955). The unit of activity was taken as (*a*) μg of tyrosine liberated in 1 hr at 37° C, (*b*) μg of benzoylarginine liberated in 1 hr at room temperature. The proteolytic activity was expressed as units/mg protein.

Estimation of protein content of plasmin preparations. The protein content was expressed in terms of bovine serum albumin. The protein was precipitated with excess of 1·7M perchloric acid and the precipitate dissolved in 0·1 N-NaOH. The ultra-violet absorption was read at 285 mμ using a Hilger Uvispek spectrophotometer and the protein content estimated from a standard curve for bovine serum albumin.

Materials

Human plasmin. (1) 'Mullertz plasmin' was prepared according to the method of Mullertz (1955), using citrated human plasma obtained from the Edgware transfusion centre. (2) Human plasmin (Merck) was kindly supplied by Merck, Sharpe and Dohme Ltd.

Bovine plasmin was a gift of Parke, Davis Company.

Human plasminogen was prepared by the method of Kline (1953) the final solution being freeze-dried in 2 ml. ampoules and stored at −10° C. Citrated human plasma from the Edgware transfusion centre was used in its preparation.

Streptokinase. Streptokinase-Streptodornase (Burroughs Wellcome).

Trypsin. Crystalline trypsin (Armour Ltd.).

Casein solution (3%) was prepared from goat casein by the method of Mullertz (1955).

Benzoylarginine ethyl ester was kindly prepared by Mr A. Hemmings of the National Institute for Medical Research.

Pseudo-globulin solution was prepared from dog plasma and consisted of the fraction of plasma protein precipitated by $(NH_4)_2SO_4$ 33–46% saturation. This precipitate was dialysed for 48 hr against running tap water and the final solution (one-third of the original volume) freeze-dried in 2 ml. ampoules and stored at −10° C.

Bradykinin was kindly supplied by Dr W. Beraldo.

RESULTS

Formation of a smooth muscle stimulating substance by plasmin

For determining the development of smooth muscle stimulating activity by the action of plasmin, the pseudo-globulin fraction of plasma proteins was used as substrate. Plasma itself was not suitable as substrate, as most samples of plasma contain an inhibitor of plasmin. In the case of human plasma even the pseudo-globulin fraction contains plasmin inhibitor, so that usually pseudo-globulin prepared from dog's plasma was used as substrate. When samples of plasmin prepared by Mullertz's method were incubated with this pseudo-globulin the incubated mixture acquired smooth muscle stimulating activity. The development of this activity in incubated mixtures of human plasmin and dog pseudo-globulin is shown on the guinea-pig's ileum in Fig. 1. The activity of the incubated mixture was not due to either histamine or acetylcholine, as the contractions were unaffected by the presence of the antagonists mepyramine or atropine. However, the response of the guinea-pig's ileum closely resembled that of the polypeptide bradykinin in being a slow contraction.

The experiment of Fig. 1 also illustrates that as incubation proceeded the smooth muscle stimulating activity of the mixture increased, reaching a maximum after about 20 min incubation at 34° C. More prolonged incubation resulted in the gradual decrease of activity which eventually disappeared altogether. This is exactly what happens when trypsin is incubated with the same substrate to form bradykinin.

Fig. 1. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*) and (*j*) bradykinin 10 μg. Plasmin 4 mg (*b*) caused no contraction. Mixture of plasmin 4 mg and pseudo-globulin solution 0·2 ml. incubated for 2 min at (*c*), 5 min at (*d*), 10 min at (*e*), 15 min at (*f*), 20 min at (*g*), 25 min at (*h*) and 40 min at (*i*).

The disappearance of the smooth muscle stimulating activity in a mixture of trypsin and pseudo-globulin or of plasmin and pseudo-globulin does not result from the destruction of bradykinin by trypsin or plasmin but from the presence in the pseudo-globulin fraction of a bradykinin-destroying factor. Evidence of this is shown by the fact that if bradykinin itself is incubated with the pseudo-globulin solution its smooth muscle stimulating activity gradually diminishes and eventually disappears.

The striking similarity between the formation and deterioration of the smooth muscle stimulating activity of incubated mixtures of trypsin and pseudo-globulin on the one hand and plasmin and pseudo-globulin on the other suggested that in both instances the smooth muscle stimulating activity might be due to the same plasma kinin—bradykinin.

The human plasmin made by Merck, Sharpe and Dohme Ltd. behaved like Mullertz's plasmin when incubated with pseudo-globulin, producing smooth muscle stimulating activity which increased with time of incubation and reached a maximum after 20 min incubation at 34° C.

Bovine plasmin (Parke, Davis) when incubated with pseudo-globulin under the same conditions did not cause contraction of the smooth muscle preparations. This, however, does not necessarily mean that a plasma kinin was not formed, because even if it were formed it would not have contracted the smooth muscle since bovine plasmin itself renders these preparations insensitive. For example, the guinea-pig's ileum did not respond to histamine, 5-hydroxytryptamine, substance P or bradykinin for more than 1 hr after 5 mg of bovine plasmin was added to the 15 ml. organ bath for 5 min. In contrast it was observed that both human plasmin and streptokinase potentiated the responses of the guinea-pig's ileum to bradykinin.

Fig. 2. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. Mixture of plasminogen 0·5 mg, streptokinase 1000 units and pseudo-globulin solution 0·1 ml. incubated for 2 min at (*a*), 5 min at (*b*), 10 min at (*c*), 15 min at (*d*) and 20 min at (*e*). At (*f*) plasminogen and pseudo-globulin solution 0·1 ml. and at (*g*) streptokinase 1000 units and pseudo-globulin solution 0·1 ml., each incubated for 20 min.

The precursor of plasmin, plasminogen, prepared from human plasma, did not give rise to any smooth muscle stimulating activity when incubated with pseudo-globulin solution. But when streptokinase, which activates plasminogen to plasmin, was added to a mixture of plasminogen and pseudo-globulin the mixture caused a contraction of isolated smooth muscle. This is shown in the experiment of Fig. 2 on the guinea-pig's ileum. As in the experiment in which plasmin was incubated with pseudo-globulin, the smooth muscle stimulating activity of an incubated mixture of plasminogen, streptokinase and pseudo-globulin increased with time of incubation and reached a maximum after 20 min incubation. No smooth muscle stimulating activity developed when streptokinase and plasminogen or streptokinase and pseudo-globulin were incubated for 20 min.

Comparison of the plasma kinin formed by plasmin with bradykinin

A detailed comparison was made of the active principle isolated from an incubated mixture of plasmin and pseudo-globulin solution with a standard preparation of bradykinin. The plasma kinin caused the same type of response as bradykinin when added to smooth muscle preparations which respond biphasically to bradykinin. The experiment of Fig. 3 shows the biphasic responses of the isolated rat's colon to the plasma kinin prepared from plasmin and pseudo-globulin, to bradykinin, and to another smooth muscle stimulating polypeptide, substance P.

The response of the guinea-pig's ileum to bradykinin is a slow contraction, preceded by a short latency and followed by a slow relaxation. Fig. 4 illustrates the similarity in the responses of the guinea-pig's ileum to both the plasma kinin and to bradykinin and the different, more rapid, response to substance P. Further, both the plasma kinin and bradykinin cause contraction of the isolated rat's uterus. The responses of this tissue to bradykinin are characterized by the fact that the latency period is directly proportional to the amount of substance added. Such a latency also occurred when the plasma kinin formed by plasmin was added to the organ bath containing a rat's uterus.

Fig. 3. Rat's colon suspended in 15 ml. of de Jalon's solution. Time marker, 30 sec. At (*a*) powder prepared from incubated mixture of plasmin and pseudo-globulin, 4 mg; at (*b*) bradykinin 300 μg; at (*c*) substance P 100 μg.

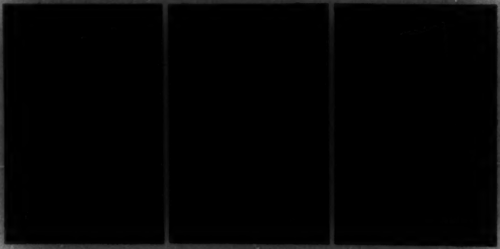

Fig. 4. Guinea-pig's ileum suspended in 15 ml. of Mg-free Tyrode solution. Time marker, 30 sec. At (*a*) powder prepared from incubated mixture of plasmin and pseudo-globulin, 0·5 mg; at (*b*) bradykinin 15 μg; at (*c*) substance P 150 μg. *W* indicates that the tissue was washed by allowing the bath to overflow.

When injected arterially into the skin of a cat's hind limb both substances produce a pronounced vasodilatation. The experiment of Fig. 5 illustrates this and also shows the response to substance P under the same conditions.

Quantitative comparison

Although qualitatively the actions of the plasma kinin prepared from plasmin and pseudo-globulin were similar to those of bradykinin in all tests, a quantitative comparison on several tissues is desirable in helping to establish the identity of the plasma kinin as bradykinin. In order to show the value of this quantitative test more clearly, the sensitivity of four tissues to the plasma kinin, to bradykinin and for comparison, to substance P as well, was estimated and the results are shown in Table 1. This table gives the amounts of bradykinin and substance P which have equal activity with 1 mg of the plasma kinin from plasmin when all three substances are tested under the same conditions on the rat's uterus, the rat's colon, the guinea-pig's ileum and the blood flow through the skin of the cat's hind limb. Each figure is the average of three experiments. The results show clearly that within the limits of experimental error the sensitivity of these preparations to the plasma kinin and bradykinin varies in an identical manner, whereas in the case of substance P there are large discrepancies.

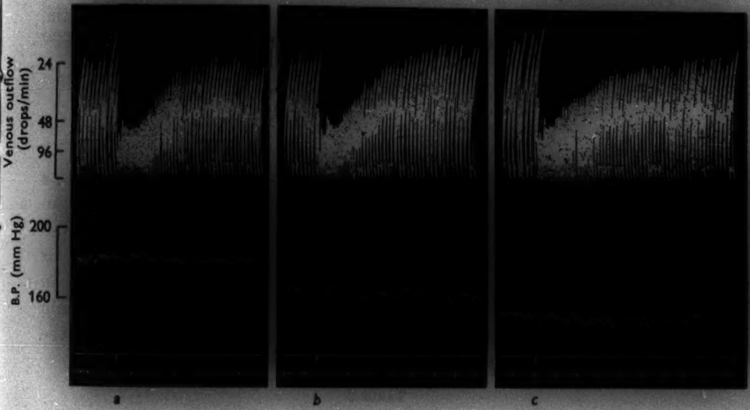

Fig. 5. Cat 2·7 kg, anaesthetized with pentobarbital 40 mg/kg. Upper tracing record of venous outflow from the skin of the hind limb. Lower tracing, arterial blood pressure. Arterial injection of bradykinin 80 μg at (*a*), of the powder made from plasmin and pseudo-globulin 0·8 mg at (*b*), and of substance P 100 μg at (*c*). Time marker, 30 sec.

TABLE 1. Amounts of bradykinin and substance P of equal activity with 1 mg of the powder, prepared from an incubated mixture of plasmin and pseudo-globulin

Tissue	Bradykinin (μg)	Substance P (μg)
Guinea-pig's ileum	35	377
Rat's uterus	37	3750
Rat's colon	53	11
Vasodilatation in cat's skin	60	150

Stability

The plasma kinin prepared from plasmin and pseudo-globulin solution behaved like bradykinin when subjected to simple chemical tests. Activity was unaffected by boiling for 1 hr in neutral or acid solution (N-HCl), but disappeared after boiling for a few minutes in 0·1 N-NaOH. In addition, both plasma kinins were destroyed after 1 hr incubation with 100 μg chymotrypsin, but not when incubated with 100 μg of trypsin for 1 hr.

Plasma kinin-forming activity of the plasmin preparations

In order to examine quantitatively the plasma kinin-forming activity of the plasmin preparations and of activated plasminogen, they were incubated with excess of substrate at 34° C for 20 min, the mixtures then assayed against a standard bradykinin on the isolated guinea-pig's ileum, and the plasma kinin-forming activity was expressed as micrograms of bradykinin formed per milligram of protein. It was found with this method that the plasmin preparations and activated plasminogen from human plasma were far less active than crystalline trypsin, as is shown in Table 2. The estimated amounts of bradykinin in the incubated mixtures err on the low side because of the presence in the pseudo-globulin fraction of an agent which destroys bradykinin.

TABLE 2

	Proteolytic activity (units/mg protein)	Esterolytic activity (units/mg protein)	Plasma-kinin-forming activity (μg/mg protein)
Crystalline trypsin	73,400	440,400	268
Bovine plasmin (Parke, Davis)	126	815	—
Human plasmin (Merck)	232	1,730	24
Human plasmin (Mullertz)	185	1,250	17
Human plasminogen + streptokinase	617	7,590	40

Proteolytic action

Although the plasmin and activated plasminogen preparations were less active when assayed by the non-specific hydrolysis of casein than by the more specific hydrolysis of synthetic benzoylarginine ethyl ester, the proteolytic and esterolytic activities of the enzyme preparation relative to those of crystalline trypsin run almost parallel. When human plasminogen was tested for proteolytic and esterolytic activity it was found to be almost inactive. However, when streptokinase 500 μ/ml. was added to the mixture of plasminogen and substrate, proteolytic and esterolytic activities developed.

It can be seen from Table 2 that trypsin, which is most active as a proteolytic enzyme, is also most active as a bradykinin-forming enzyme, and similarly for the less active human plasmin preparations the proteolytic and plasma kinin-forming activity run parallel. For bovine plasmin it was not possible to determine its plasma kinin-forming activity because of its inhibitory action on smooth muscle. It is interesting that the ratio between the proteolytic activities of the human plasmins and of crystalline trypsin is considerably higher than the ratio between the plasma kinin-forming activities of the plasmins and of trypsin. This suggests that, given a preparation of plasmin with a proteolytic activity equivalent to crystalline trypsin, its plasma kinin-forming activity would be considerably greater than that of crystalline trypsin.

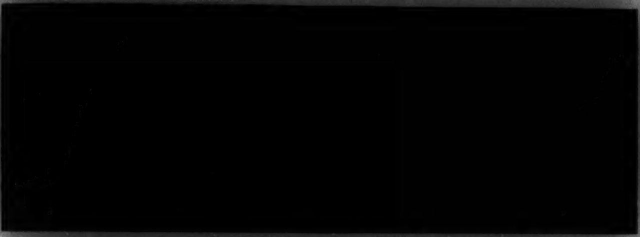

Fig. 6. Guinea-pig's ileum suspended in 15 ml. of Mg-free Tyrode solution. At (*a*), (*c*) and (*e*) plasmin (Merck) 1 mg and pseudo-globulin solution 0·1 ml., incubated for 20 min at 34° C. At (*b*) soya bean trypsin inhibitor 100 μg and at (*d*) antiplasmin 3 mg were added to the mixture of plasmin and pseudo-globulin before incubation.

Inhibition of the plasma kinin-forming activity of plasmin by antiplasmin

Plasma is known to contain a proteolytic inhibitor (Hahn, 1897) which has been isolated from plasma and purified and is known as antiplasmin (Loomis, Ryder & George, 1949). Antiplasmin as well as another inhibitor of proteolytic enzymes, soya bean trypsin inhibitor, was found to inhibit the formation of plasma kinins when plasmin is incubated with pseudo-globulin. The result of such an experiment is illustrated in Fig. 6, which shows the response of the guinea-pig ileum to incubated mixtures of plasmin and pseudo-globulin alone (*a*, *c* and *e*) and in the presence of soya bean trypsin inhibitor (at *b*) and of antiplasmin (at *d*). Both antiplasmin and soya bean trypsin inhibitor also inhibited the formation of the smooth muscle stimulating activity in an incubated mixture of plasminogen, streptokinase and pseudo-globulin.

Plasma kinin-forming activity of plasma

Whole blood or plasma usually contains sufficient of the inhibitors of plasmin and of plasminogen activator to hold the proteolytic system in check. Under certain conditions the equilibrium in this system can be upset and the proteolytic activity of plasma then increases. For instance, plasma treated with chloroform develops proteolytic activity (Christensen, 1946) and exhibits smooth muscle stimulating activity which untreated plasma does not possess. However, when excess antiplasmin is added to plasma before it is treated with chloroform it does not develop this smooth muscle stimulating activity. The experiment of Fig. 7 illustrates the smooth muscle stimulating activity which develops in plasma treated with chloroform, but which does not occur when an excess of antiplasmin has first been added to the plasma.

Smooth muscle stimulating activity also appears when plasma comes into contact with glass (Armstrong *et al.* 1957). It seems likely that contact with a glass surface might activate the precursor of plasmin, particularly since Rapaport, Aas & Owren (1955) have shown several enzymes involved in blood clotting to be activated in this way. If this were so, it would be expected that the activation would be inhibited by antiplasmin. In fact, the smooth muscle stimulating activity which develops when plasma is transferred from a siliconed glass vessel to an unsiliconed vessel was readily inhibited by addition of antiplasmin. This is illustrated in the experiment of Fig. 8. At *a* and *b* are shown the small contractions of the guinea-pig ileum following addition of 0·3 ml. of freshly drawn human plasma which had been standing in a siliconed glass vessel for 1 and 5 min respectively, and at *c* and *d* are the stronger responses to 0·3 ml. of the same sample of plasma after standing in an unsiliconed glass vessel for 1 and 5 min respectively. When 0·1 ml. of saline containing 300 μg of antiplasmin was present in the unsiliconed glass vessel before allowing the plasma to stand in it then the smooth muscle stimulating activity did not appear, as shown at *e* and *f*. The glass activation of plasma is also inhibited by soya bean trypsin inhibitor (Armstrong *et al.* 1957).

Fig. 7. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*) human plasma 0·5 ml., at (*b*) and (*d*) human plasma 0·5 ml. shaken with chloroform 0·02 ml. and at (*c*) a mixture of human plasma 0·5 ml. and antiplasmin 1·5 mg shaken with chloroform 0·02 ml.

Schachter (1956) showed that plasma diluted with physiological saline developed smooth muscle stimulating activity. Dausset, Bergerot-Blondel & Colin (1956), who found that plasma also develops proteolytic activity on dilution, suggested that this proteolytic activity was due to an activation of plasmin. This suggestion is not borne out by the following observations. When freshly drawn human plasma kept in a siliconed vessel was diluted 1 to 10 with physiological saline or with Tyrode solution, it developed smooth muscle stimulating activity in 15 min at 34° C, as shown in Fig. 9. The same occurred when 1 or 3 mg/ml. of antiplasmin had been added to the plasma (Fig. 9*c*, *e*).

Fig. 8. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*) and (*b*) freshly drawn human plasma 0·3 ml. allowed to stand in a siliconed glass vessel for 1 min and 5 min respectively. At (*c*) and (*d*) 0·3 ml. portions from the same sample of plasma, and at (*e*) and (*f*) plasma 0·3 ml. plus antiplasmin 300 μg, allowed to stand in an unsiliconed glass vessel; (*c*), (*e*) for 1 min and (*d*), (*f*) for 5 min.

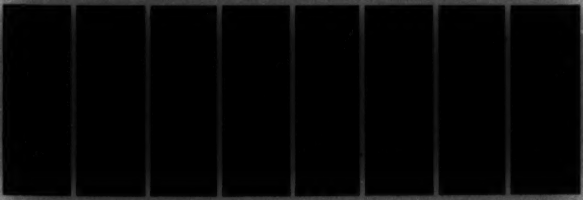

Fig. 9. Guinea-pig's ileum suspended in 15 ml. Mg-free Tyrode solution. At (*a*), (*d*) and (*h*) bradykinin 5 μg. Human plasma 0·1 ml. stored in a siliconed vessel diluted 1:10 with Tyrode solution at (*b*) and (*g*). The same plasma 0·1 ml. diluted 1:10 with Tyrode solution containing 1 mg antiplasmin at (*c*), 3 mg antiplasmin at (*e*) and 100 μg soya bean trypsin inhibitor at (*f*).

On the other hand, the addition of soya bean trypsin inhibitor 100 μg/ml. to the plasma completely inhibited the development of the activity (Fig. 9 *f*) as was shown already by Schachter (1956). The concentration of antiplasmin could not be increased above 3 mg/ml. plasma as high doses of antiplasmin caused depression of the smooth muscle.

Miles & Wilhelm (1955) and Wilhelm, Miles & Mackay (1955) have found that an α_2 globulin from guinea-pig serum, which they call permeability factor, when injected intracutaneously into guinea-pigs causes vasodilatation and increased permeability. It was of interest to test whether or not this permeability factor interacted with other plasma proteins to form plasma kinins which could account for part of this activity. Samples of the fractions G2 and G2α kindly supplied by Dr D. L. Wilhelm were tested for the formation of plasma kinins by incubation with pseudo-globulin solution. Neither of these fractions caused any contraction when added to the isolated guinea-pig ileum alone, but after 15 min incubation at 34° C with pseudo-globulin solution the G2 fraction gave rise to smooth muscle stimulating activity while the purified G2α developed no such activity under the same conditions. This is illustrated in Fig. 10, which also shows that the development of smooth muscle stimulating activity by G2 is inhibited by soya bean trypsin inhibitor but not by antiplasmin. The failure of G2α to form smooth muscle stimulating activity on incubation with pseudo-globulin cannot be explained by the presence of an inhibitor in the G2α fraction which would prevent the development of this activity. This is illustrated in Fig. 10 (at *e*) which shows that after incubation with pseudo-globulin G2 still forms smooth muscle stimulating substance in the presence of G2α.

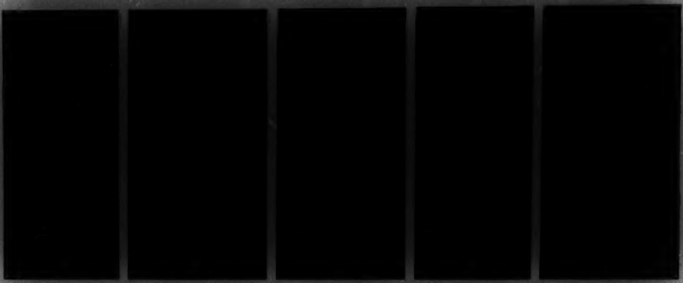

Fig. 10. Guinea-pig's ileum suspended in 15 ml. Tyrode solution. Permeability factor G2 0·3 ml. incubated with 0·1 ml. pseudo-globulin solution for 15 min, alone at (*a*), with antiplasmin 3 mg at (*b*) and with soya bean trypsin inhibitor 200 μg at (*c*). Permeability factor G2α 0·5 ml. incubated with 0·1 ml. pseudo-globulin solution for 15 min at (*d*). Permeability factor G2 0·3 ml. incubated for 15 min with 0·5 ml. G2α and 0·1 ml. pseudo-globulin at (*e*).

DISCUSSION

The present experiments show that plasmin, a proteolytic enzyme in blood, when incubated with plasma globulins, produces a plasma kinin which has vasodilator properties and stimulates isolated smooth muscle, but that the precursor of plasmin, plasminogen, has neither proteolytic nor plasma kinin-forming activity, although these activities appear when plasminogen is activated to plasmin by the specific activator, streptokinase. The finding that the inactive precursor of plasmin forms a plasma kinin only when activated by its specific activator precludes the possibility that the plasma kinin-forming activity is due to an impurity in the plasmin preparations. An impurity which could have been responsible for this activity is kallikrein and the question naturally arises, what is the relationship between plasmin and kallikrein?

Kallikrein has been described by Frey *et al.* (1950) as a depressor substance which is capable of forming kallidin (a plasma kinin which is probably identical with bradykinin) when it is incubated with plasma proteins. Although it is not certain whether all the actions of kallikrein are due to the formation of plasma kinins or whether it has a vasodilator action of its own, it is clear that kallikrein is different from plasmin. Kallikrein is thermolabile and readily destroyed by acid, whereas plasmin retains most of its activity even after boiling at pH 2 for 30 min, which is, in fact, one step in the preparation of 'Mullertz' plasmin.

There is a close analogy between blood kallikrein and plasmin. Just as kallikrein is present in blood in an inactive form so plasmin is present as its inactive precursor, plasminogen. It has been shown that when plasma is treated with chloroform there is an increase in the proteolytic activity of the plasma which has been attributed to plasmin (Christensen, 1946). In the present experiments it has been shown that when plasma is treated with chloroform there is an appearance of smooth muscle stimulating activity (like that of a plasma kinin) and the development of this activity is inhibited by the presence of excess antiplasmin. Another interesting phenomenon which appears to involve the activation of plasmin is the increase in proteolytic activity in plasma which occurs on contact with glass. Armstrong *et al.* (1957) have observed that when freshly collected plasma comes into contact with glass it develops smooth muscle stimulating activity like that of a plasma kinin. This has been confirmed in the present experiments and it has also been shown that the development of this activity is inhibited not only by soya bean trypsin inhibitor but also by antiplasmin. Armstrong *et al.* have shown that not only smooth muscle stimulating and vasodilator plasma kinins are produced under these conditions but also that pain-producing plasma kinins are formed as well. It is therefore interesting to consider the possibility that plasmin forms not only plasma kinins with these actions but perhaps other plasma kinins exerting the diverse effects which form part of the tissue reaction to injury. For instance, it is possible to envisage the formation of plasma kinins with chemotaxic activity (like the leucotaxin of Menkin, 1938) under such conditions.

Frey, Kraut & Werle found enzyme-like depressor substances in other body fluids and tissues in addition to kallikrein in blood. These were also 'dubbed' kallikrein although there is some evidence that they are different from the kallikrein of blood. One site in which such an enzymic material was found is the salivary glands and recent experiments on functional hyperaemia in the salivary gland (Hilton & Lewis, 1955, 1956) have shown that functional hyperaemia in the submandibular salivary gland is the result of vasodilator plasma kinins (probably bradykinin) formed in the interstitial fluid as a result of the escape from the activated gland cells of this enzyme.

It was observed that in order for the maximum amount of plasma kinin to be formed it was necessary to incubate the plasmin and the pseudo-globulin for about 20 min. This delayed formation is quite different from the rapid formation of plasma kinin in the salivary gland. When saliva and pseudo-globulin are incubated the maximum plasma kinin formation occurs in 1–3 min (Hilton & Lewis, 1955). There may be a functional significance in these differences in the time of development of the vasodilator activity in the two cases. Hilton and I have shown that plasma kinin formation plays an important role in the functional vasodilatation in the salivary glands. In this case it would be essential that the vasodilator material be produced rapidly in order to account for the pronounced vasodilatation which occurs when the glands are activated. However, it is more likely that kinin formation by plasmin is important not in physiological mechanisms but in pathological conditions. For instance, in cases of tissue injury it is known that substances such as histamine are released locally from the injured tissue. These substances would increase the capillary permeability, allowing the plasminogen from the general circulation to enter the interstitial fluid and to make contact with the plasminogen activators (Astrup & Permin, 1947) escaping from the damaged cells. The activated plasminogen would then proceed to form vasodilator plasma kinins like bradykinin and possibly plasma kinins having other actions. Thus the secondary reactions of tissue injury which are known to be delayed in onset would be accounted for in this way.

A second instance where activation of plasminogen may play a role in pathological conditions is in generalized vascular shock, as suggested by Beraldo (1950). Under these conditions it might be envisaged that surface effects brought about by protein interaction, possibly acting in the same way as Armstrong *et al.* (1957) have shown a glass surface to act, would lead to an activation of plasminogen, not in the interstitial fluid as in the case of a localized reaction, but in the circulating blood, so that the plasma kinins formed in the circulation might play a role in this characteristic delayed general vascular collapse.

Another condition not yet discussed under which smooth muscle stimulating activity can develop in plasma is by dilution. The finding in the present experiments that antiplasmin does not prevent the development of smooth muscle stimulating activity in plasma when it is diluted suggests that the activation by dilution is not due to activation of plasmin. It might well be that on dilution the kallikrein-inactivator complex is split as suggested by Schachter (1956) leaving kallikrein free to form plasma kinins.

Finally, the present experiments with the permeability factor of Miles & Wilhelm (1955) confirm their view that the purified factor does not act by forming plasma kinins, when it comes into contact with plasma proteins. However, it is possible that some of the effects exerted by the crude fraction G2 may be due to the presence of a plasma kinin-forming enzyme which does not behave like plasmin, as its action is not inhibited by antiplasmin but only by soya bean trypsin inhibitor.

SUMMARY

1. When plasmin, a proteolytic enzyme in blood, is incubated with plasma globulins, a plasma kinin is produced. The name plasma kinin includes all bradykinin-like polypeptides derived from plasma proteins.

2. A powder has been prepared from an incubated mixture of human plasmin and dog pseudo-globulin, containing a plasma kinin which on comparison with bradykinin was found to be indistinguishable from it.

3. Although crystalline trypsin was much more active than plasmin both as a proteolytic enzyme and as a plasma kinin-forming enzyme it was estimated that a plasmin preparation with a proteolytic activity equal to that of crystalline trypsin would be considerably more active as a plasma kinin-forming enzyme.

4. Plasmin differs from kallikrein, another enzyme in blood, in being thermostable and resistant to acid.

5. Plasminogen, the inactive precursor of plasmin, possesses neither proteolytic nor plasma kinin-forming activity, but acquires these properties on addition of the specific activator, streptokinase.

6. The development of smooth muscle stimulating activity when plasma is treated with chloroform or brought into contact with a glass surface is probably due to activation of plasminogen and the subsequent formation of a plasma kinin.

7. Activation of plasminogen does not account for the appearance of smooth muscle stimulating activity when plasma is diluted with saline, since the development of the activity is unaffected by antiplasmin.

8. The purified preparations of the permeability factor, G2α, do not form plasma kinins on incubation with plasma proteins, although the crude fraction G2 does contain a plasma kinin-forming enzyme.

9. It is suggested that plasmin plays a role in local tissue reactions when plasminogen is activated in the interstitial fluid with subsequent delayed formation of plasma kinins.

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RESTING POTENTIALS OF DIAPHRAGM MUSCLE AFTER PROLONGED ANOXIA

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Experiments on isolated mammalian tissue at body temperature require vigorous oxygenation in most cases, for these tissues are usually fairly thick and their oxygen consumption is high. In many situations there is some doubt regarding the adequacy of the supply of oxygen. It is shown in the present study that exploration of muscle by the use of micro-electrodes may be used to provide a criterion of adequate oxygenation, for anoxia characteristically produces lowered resting potentials in the depth of the muscle. The findings may also be extended to give some information regarding oxygen requirements and oxygen diffusion through mammalian muscle at body temperature. In addition, certain physical dimensions of the tissue, including thickness, mean cell diameter and interfibre space, may be estimated from the results of the experiments.

METHODS

Saline solution. The composition was (mM): Na^+ 145, K^+ 5·0, Ca^{2+} 1·2, Mg^{2+} 1·2, Cl^- 137·5, HCO_3^- 12·5, SO_4^{2-} 1·2, HPO_4^{2-} plus $H_2PO_4^-$ 1·2. The glucose content was 100 mg/100 ml. The solution was gassed with a mixture containing 2% (v/v) carbon dioxide and variable oxygen content, and the pH was 7·5 at 37° C.

Handling of tissues. Left diaphragm muscles from rats of 150–250 g were used for measurement of resting potentials. It was necessary to strip the pleura from the muscle before impaling with micro-electrodes. This was facilitated during dissection by removal of a lower lobe of the left lung, which was often invaginated between the diaphragm and part of the heart. The pleura, which was here loose, could then be seized with forceps and stripped from the muscle, the process being started near the central tendon and proceeding towards the rib margin.

The hemidiaphragm was attached in a vertical position to a plastic frame which was partly immersed in an organ bath filled with saline (Fig. 1). The tendon was tied to the lower cross-piece of the frame, while the ribs were attached to threads which passed up through holes in the frame and hence to a weight of 25–50 g, which was required to keep the muscle sufficiently steady for impalement by micro-electrodes. The muscle was arranged so that the pleural surface faced the electrode. The plastic organ bath was made with rectangular faces and was jacketed on two sides, the saline being maintained at 37° C by water which was circulated from a heated reservoir by a centrifugal pump. One wall of the organ bath was polished, and this enabled the surface fibres of the muscle to be viewed by means of a dissecting microscope. Dissections and other manipulations were performed in a shallow bath containing saline which was continuously oxygenated.

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Oxygenation. Two gas mixtures were obtained, one containing 2% (v/v) carbon dioxide and 98% oxygen, the other containing 2% carbon dioxide and 98% nitrogen. The contents of the two cylinders were allowed to mix in suitable proportions and the gas was then led to the saline through a distributor, formed from a length of plastic tube whose end had been plugged with glass wool dipped in chloroform and clamped. This produced a fine cloud of gas bubbles which enveloped the tissue. Auxiliary gas jets were used to ensure that all parts of the saline were well aerated. The inflowing gas was analysed from time to time by means of a magnetic oxygen analyser, and the oxygen content in the mixture was adjusted if necessary. Samples of the gas mixture were also withdrawn by a needle and syringe from a short length of thick-walled rubber tubing which led to the organ bath, and the contents were analysed by conventional manometric methods.

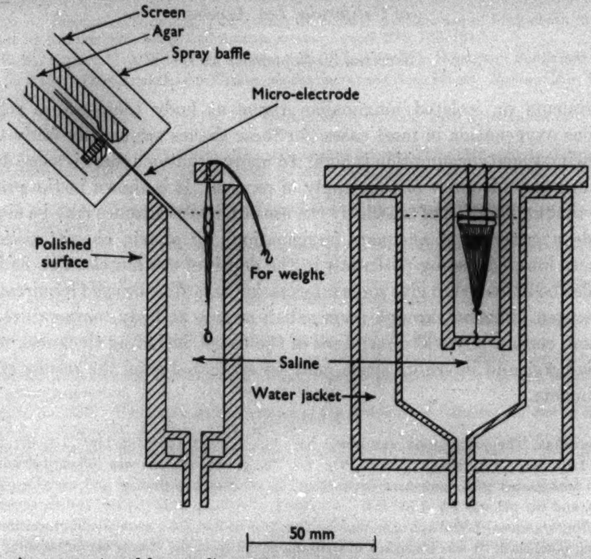

Fig. 1. Arrangement used for recording resting potentials. Two sections at right angles are shown. The muscle was held vertically, and the micro-electrode approached at an angle of 42° to the tissue. The electrode was driven slowly through the muscle by a motor. The gas distributor, which produced a fine cloud of gas bubbles around the tissue, has been omitted. One wall of the plastic bath was polished, so that the muscle could be viewed by a microscope.

Micro-electrode technique. The methods in general resembled those of Nastuk & Hodgkin (1950). The electrodes were 7 cm long, one end being clamped in a plastic holder (Fig. 1), so that contact was made with the agar jelly (1·5% with 2·5M-KCl), which also contained the end of a short chlorided silver wire which was soldered to the control grid of the first valve. The electrode holder and cathode-follower circuit were attached to the rack taken from a microscope fitted with the usual mechanism for fine adjustment, and the whole was mounted on a universal adjustable milling table. By this means the electrode could be advanced or withdrawn or moved from side to side.

The electrode approached the muscle at an angle of 42° with the vertical, and could be slowly driven down and through the substance of the tissue at a rate of 0·37 mm/min by a synchronous motor which was used to rotate the pinion of the fine adjustment mechanism.

The cathode-follower input circuit was formed from a miniature pentode, Raytheon CK 5886, and both the heater and the high tension were supplied by small mercury batteries. This valve had an anode current of $6\mu A$, and in conditions of low humidity the grid current was below 10^{-14} A, as measured by inserting a grid resistance of $10^{12}\,\Omega$. In humid conditions the grid current was approximately 10^{-12} A. The cathode resistance was 650 kΩ, and in response to a signal this resistance produced a long time constant which was not a disadvantage for the present measurements. The output of the cathode-follower stage was led to an amplifying pen recorder having a high input impedance and a rise time of approximately 0·4 sec for a deflexion between 5–95% of the range. An oscilloscope with a d.c. amplifier was also used as a monitor.

Electrodes were prepared from capillary tubing (Kimble Glass Co.) of outside diameter 0·7 mm and wall thickness 0·2 mm, a machine similar to that described by Alexander & Nastuk (1953) being used. Careful selection of electrodes was made and only those having resistances between 10 and 30 MΩ were retained. Results obtained by penetration of muscle fibres were only accepted if no change in the resistance of the electrode occurred and if there was no change in the base line of the oscilloscope or pen recorder (see later). Some batches of electrodes gave frequent sudden increases of recorded potentials accompanied by an increase in the electrode resistance, as described by del Castillo & Katz (1955) and by Adrian (1956); results from these electrodes were discarded. The recording system was calibrated by means of a Weston cell.

Precautions were necessary to ensure that spray did not reach the recording system and produce spurious potential changes. For this reason the electrodes were long, and during recording the bath and contents were covered with a cellophane wrapper having a slit for the electrode. At times it was necessary to blow away the spray by means of an air jet directed on to the exposed shaft of the electrode.

Measurement of linear dimensions. Horizontal movement of the pen recorder (see Fig. 2) was related to the horizontal component of the movement of the electrode through the tissue by measurement of: (*a*) the relation between movement in the direction of the electrode shaft with rotation of the pinion of the microscope holder (which carried the electrode assembly); this was 185μ/rev; (*b*) the speed of the synchronous motor, which was in most cases 2 rev/min; (*c*) the angle θ between the shaft of the electrode and the vertical muscle (42°, so that $\sin\theta$ was 0·70); and (*d*) the speed of the paper used for the pen recorder, which was 2 in. (51 mm)/min. Movement in a horizontal plane through the tissue was magnified by a factor of 205.

The thickness was obtained from records similar to that of Fig. 2 by measuring between the first and the last full deflexion. Extrafibre space was obtained by measuring the intervals between deflexions and expressing the sum as a fraction of the thickness. Only records which were technically satisfactory (see above) were used.

In some cases the cell thickness was also measured at approximately the mid point of the deflexion. No correction was made for the lag of the pen recorder, which corresponded to a movement of the electrode of about 2μ for a full-scale deflexion. It was considered that any error recorded in piercing the cell would be balanced on leaving it. Similar considerations may be applied to possible denting of the cell membrane. In general, however, the electrode passes not through the diameter of the fibre but through a chord, and the thickness as recorded will be smaller than the true value and must be corrected. The distribution of these apparent values is also expected to show marked skewness, and this problem is analysed in an Appendix (see below) which also gives methods of applying corrections to the results.

Measurement of oxygen consumption. Oxygen consumption was measured in independent experiments by a method which was similar to that described by Whalen (1956, 1957). Diaphragm muscles were secured in small plastic vessels whose total volume, including manometers, was 11 ml. The vessel contained 8 ml. saline and the contents were saturated with a mixture of 2% carbon dioxide and 98% oxygen. The consumption was estimated manometrically, and during runs the concentration of carbon dioxide in the space above the saline was maintained at 2% by the use of diethanolamine (Krebs, 1951). A magnetic stirrer rotated inside the saline and caused sufficient turbulence and bubble formation to aerate the muscle. The whole vessel was immersed in a water-bath at 37° C. The tension on the muscle was adjusted to 25 g wt. by a thread which was attached to the tendon and passed upwards through a mercury seal to a strain gauge mounted on a rack.

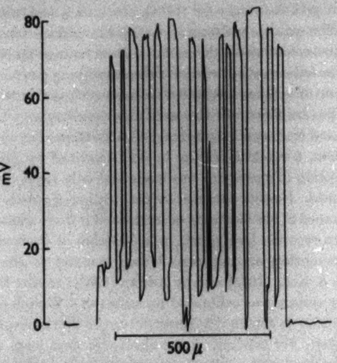

Fig. 2. Potential changes recorded by a pen writer during penetration of micro-electrode through normal diaphragm muscle from right (pleural surface) to left (peritoneal surface). Upward deflexion indicates a potential at the electrode tip which is negative to the earthed saline. The deflexions are presumably due to entry into and exit from the fibres which are encountered.

RESULTS

Penetration of normal muscle

Fig. 2 shows the record obtained when the micro-electrode was steadily propelled through diaphragm muscle by means of a motor. Penetration was from right to left, from the pleural to the peritoneal surface, and took over 2 min for completion. Entry into the tissue was accompanied by a series of deflexions of the pen recorder in a direction such that upward movement indicated a negative potential relative to the earthed saline in the bath. The potentials were of the order of 70–80 mV and were presumably produced by penetration of the electrode into, through and out of muscle fibres which were encountered. The figure shows some fifteen deflexions. This seems to be somewhat less than the number of cell layers seen previously in transverse sections of diaphragm (Creese, 1954), but is in agreement with the smaller thickness of the muscles used in the present study (see below).

The pen writer does not return completely to the base line between deflexions, for reasons which are not clear. The electrodes frequently broke if they were pushed through the peritoneum on the far side of the muscle. When deflexions corresponding to fibres ceased, the motor was stopped and was then reversed to bring the electrode back into saline. The base line was again recorded, and this explains the break in the record at the left-hand side of Fig. 2. Changes in the base line or in the electrode resistance were attributed to plugging of the tip (Adrian, 1956) and the records were usually discarded. A second penetration at exactly the same position showed a series of low potentials, presumably due to damage produced by the passage of the electrode. If the electrode was moved a few cells away in a lateral direction, normal records could again be obtained, and with a suitable electrode the process could be repeated many times. In some cases the first deflexion, on the extreme right-hand side of the records, was markedly lower than normal. This corresponds to the cell layer immediately beneath the pleura. It is likely that the underlying cells were sometimes damaged by removal of the pleural membrane. It was necessary to put the muscles under tension to keep them sufficiently steady for impaling, and the effects here reported have all been obtained on stretched muscle. The effects of stretching have been summarized by Ling & Gerard (1949*a*), who detected no effects in the resting potential in the case of normal amphibian muscle. Irregularities in the base line were mainly caused by the vigorous direct aeration which was employed.

Muscle thickness, extrafibre space, and fibre diameter

The thickness of left diaphragm muscles from rats weighing 200–280 g ranged from 419 to 630μ, with a mean value of 500μ (twenty-seven penetrations from five muscles). The distance was measured from the first to the last deflexion, and the contribution made by the membranes was not taken into account.

These measurements, obtained on stretched muscle, resembled the values of 400–500μ recorded by Pearson, Hsieh, Dutoit & Hastings (1949). Higher values of 600μ were recorded by Holmes, Jenden & Taylor (1951), and 590μ by Creese (1954) in rats of 120 g. It is clear that there is considerable local variation.

The extrafibre space was estimated by measuring the intervals between the deflexions and expressing the sum as a fraction of the thickness. The fraction by volume occupied by the extrafibre space ranged from 0·23 to 0·39, the mean being 0·28 ± 0·014 (S.E. of seventeen measurements from nine diaphragm muscles). Results from frog sartorius muscle, obtained by methods similar to those used by Blum, Creese, Jenden & Scholes (1957), give a value of 0·19 ± 0·003 (S.E. of 7; range 0·17–0·21).

The apparent cell diameter was obtained by measuring the distance between the upward and downward movement of each deflexion in records similar to that of Fig. 2, and some 284 cells from twelve diaphragm muscles from rats of 200–300 g were measured. The distribution was markedly skewed in a positive direction. The skewness, as indicated by the ratio (3rd moment about mean): (standard deviation cubed), was +1·12. This type of skewed distribution is to be expected from the manner in which the measurements were made (see below). The mean value as measured on the records was 26·7 ± 15·7μ (S.D. of individual measurements). These figures require corrections which allow for the systematic biasing of the results caused by the arrangement whereby chords are measured instead of diameters. The corrections to be applied are considered later in an Appendix. The corrected mean diameter was 32μ in stretched muscles from rats of 200–300 g. This may be compared with a value of about 20μ obtained from histological sections of soaked diaphragms from rats of 120 g (Creese, 1954).

Effects of poor oxygenation

A stream of gas bubbles flowing past the muscle may hinder manipulations with micro-electrodes. During certain manoeuvres the oxygen supply was reduced to a trickle or was cut off for various periods of time. After such procedures it was noticed that the potentials recorded from the centre of the tissue were often below normal. Fig. 3 is an example, and shows a region containing low potentials in the middle of the muscle.

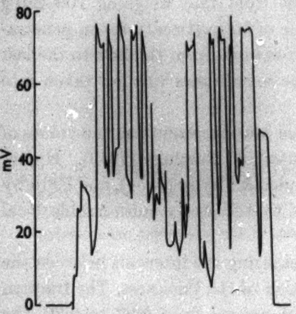

Fig. 3

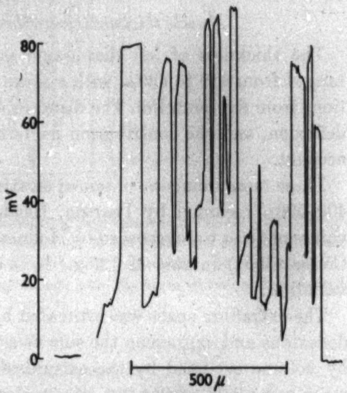

Fig. 4

Fig. 3. Record obtained as before, from muscle which had been intermittently deprived of oxygen. The cells in the centre are depolarized.

Fig. 4. Record from muscle aerated by a trickle of oxygen on the left (peritoneal) surface only. The region of depolarization is asymmetrical.

Fig. 4 shows a record obtained after a trickle of oxygen (containing 2% of carbon dioxide) had been allowed to play on the peritoneal side of the muscle (left side in Fig. 4) for 2 hr. There is a gap in the record which shows low potentials, and this time the region is asymmetrical in position, being shifted towards the side which has had least oxygen. There was apparently enough oxygen in the saline over the pleural surface (right side in Fig. 4) to provide normal potentials in the case of the first few layers of cells.

When low concentrations of oxygen were used it was found that cells in the depths of the tissue might take up to 2 hr for depolarization. Superficial cells were rapidly depolarized by a mixture of 2% carbon dioxide and 98% nitrogen, and recovery was sometimes seen, as found by Boyd & Martin (1956). These effects of anoxia appeared to be reached much more quickly than in the amphibian muscle studied by Ling & Gerard (1949*b*).

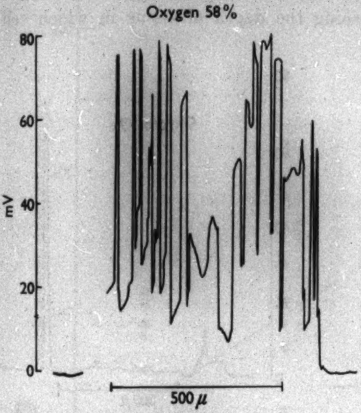

Fig. 5

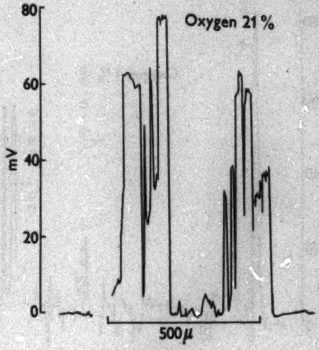

Fig. 6

Fig. 5. Potentials from muscle aerated for 3 hr with a gas mixture containing 58% oxygen. There is a region of depolarization in the centre. The low potentials of cells on the right (pleural surface of diaphragm) may be due to damage produced during stripping of the pleura.

Fig. 6. As before, using 21% oxygen for 3 hr. The depth within which resting potentials are found is narrowed.

Quantitative effects of oxygen variation

Fig. 5 shows the effect of aerating the tissue for 3 hr with a mixture containing 58% oxygen and 2% of carbon dioxide, the balance being nitrogen. Potentials are reduced in the centre of the muscle. Fig. 6 shows the effect of 21% oxygen; the gap indicating absent or reduced potentials is very obvious. Repeated penetration of the middle third of the muscle revealed no apparent change after 2 hr, and in practice at least 3 hr were allowed for a steady state to be produced in each case. The solution was changed at intervals so that the external ionic environment was approximately constant, and this procedure would also serve to maintain a constant composition for the intercellular fluid.

Fig. 7 demonstrates the effect of reducing the oxygen content to 1% for 3 hr, the carbon dioxide concentration being maintained at 2% as before. Only the outer few cells now show measurable potentials. Frequent blocking of the micro-electrode occurred when it was pushed through anoxic muscle, and the break in the record of Fig. 7 is attributed to this. In a majority of cases the outer cell shows a high potential, which suggests that a partial pressure of

7 mm Hg, corresponding to 1% oxygen in the gas mixture, is normally sufficient to maintain cellular function. In a few records a resting potential was not detected in the outer cell, and Fig. 8 shows an example of this.

The results suggest that lowering the oxygen content of the gas used to aerate the saline has the effect of diminishing the depth of tissue in which cell potentials could be recorded.

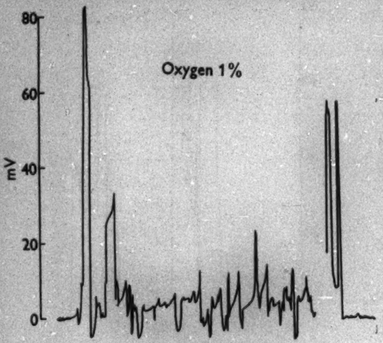

Fig. 7

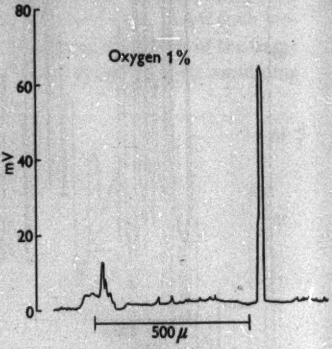

Fig. 8

Fig. 7. Record obtained with 1% oxygen, with thick muscle. Only the outer cells show resting potentials. The break in the record was caused by removal of an artifact (see text).

Fig. 8. Record from another muscle aerated for 3 hr with 1% oxygen.

The relation between these two variables was examined in six diaphragms. Each muscle was set up in 98% oxygen (and 2% carbon dioxide), and two normal records were obtained by penetration with a micro-electrode. The oxygen content was then suitably reduced, the carbon dioxide concentration being maintained constant as described above. After 3 hr several recordings were made in the middle third of the muscle. The oxygen concentration was usually lowered again for another period of 3 hr and further recordings were taken.

The records were later measured and the depth of tissue which maintained resting potentials was estimated. Measurements were made from the first deflexion and included cells having potentials greater than an arbitrary value of 15 mV. Two values were obtained from each record in all but one muscle. In any particular record a decision had to be made regarding the inclusion or otherwise of a single cell, and the estimations therefore resembled those obtained from an 'all or none' or quantal response (Gaddum, 1933). In a few records similar to the left side of Fig. 8, the critical thickness was taken to be zero. Measurements were converted to microns by the calibration factor.

The results are shown in Fig. 9. For reasons which are considered in the following paragraph, the depth in which potentials are found is plotted against the square root of the oxygen concentration. The points suggest that the critical depth, as measured above, is related to the concentration of oxygen which is in contact with the muscle.

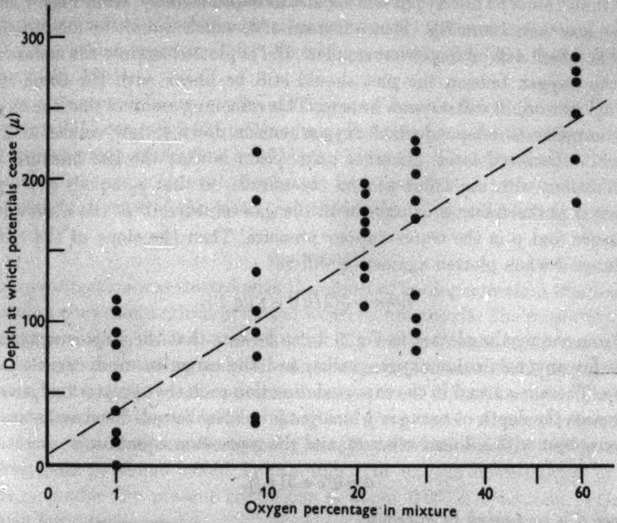

Fig. 9. Scatter diagram showing the depth at which potentials cease (or become very small) plotted against the square root of the oxygen fraction in the gas mixture. For convenience the abscissa shows values of the oxygen percentage. The broken line gives the regression. Some points represent more than one pair of values.

Interpretation of quantitative results

Oxygen reaches the depths of the muscle by a process of diffusion, and the partial pressure is progressively reduced towards the centre by oxygen consumption. If this consumption were independent of oxygen tension, then the curve relating oxygen tension to the distance from the surface would be parabolic, and the depth b at which the oxygen tension becomes zero would be

$$b = \sqrt{(2Dy_0/a)}, \tag{1}$$

where y_0 is the external oxygen tension, a is the oxygen consumption in c.c. (s.t.p.)/c.c. wet tissue/min, and D is the Krogh diffusion coefficient of oxygen through the tissue. The nomenclature is similar to that of Hill (1928). The use of the Krogh coefficient has recently been discussed by Rehberg (1955).

If the depth b could be measured for various oxygen concentrations and plotted against the square root of the oxygen concentration, eqn. (1) shows that a straight line through the origin should result, provided the above conditions are fulfilled. The measurements of the critical depth d, obtained from records similar to Figs. 5–8, give the depth at which the potential has fallen to less than 15 mV. The oxygen tension at this depth is likely, from Figs. 7 and 8, to be less than 7 mm Hg. Hence instead of b, which cannot be measured, we plot d, which will be somewhat smaller. If d is plotted against the square root of the oxygen tension the plot should still be linear with the same slope, though the origin will not now be zero. This reasoning assumes that the oxygen consumption is independent of oxygen tension down to low values, and this point is discussed later. Another assumption is that the gas mixture is in equilibrium with the saline around the muscle, so that y_0 equals $q\,(\mathrm{P}-\mathrm{p})/\mathrm{P}$ where q is the fraction of oxygen in the gas mixture, P is the atmospheric pressure and p is the water vapour pressure. Then the slope of the critical distance d when plotted against $\sqrt{q}$ will be

$$\text{slope} = \sqrt{\{2D(\mathrm{P}-\mathrm{p})/a\,\mathrm{P}\}}. \tag{2}$$

From the results plotted in Fig. 9 it can be seen that the scatter is considerable for any particular oxygen value, and the range in most cases exceeds $100\,\mu$. There is a trend in the expected direction such that greater oxygenation increases the depth of tissue in which potentials are found. The results are not inconsistent with a linear relation, and the regression equation is

$$d = 6{\cdot}7 + 311\sqrt{q}, \tag{3}$$

where d is measured in microns.

The standard error of the regression coefficient is ± 26, obtained from 54 pairs of measurements. A slope of this magnitude is unlikely to be fortuitous ($t \simeq 12$, $P < 0{\cdot}001$). The correlation coefficient is $+0{\cdot}85$. We expect the intercept on the ordinate to be negative; in fact it has a small positive value of $+6{\cdot}7$. The standard error of this predicted value is $\pm 11{\cdot}6$, so the value obtained has little significance. The permissible thickness consistent with adequate oxygenation is given by $2d$, and from eqn. (3) the maximum value, obtained with 100% oxygen with q equal to unity, is $635\,\mu$. This is similar to critical values obtained in various tissues by other methods as summarized by Field (1948).

Oxygen consumption and diffusion coefficient

Left diaphragms were used from rats of 150–200 g. In the presence of 2% carbon dioxide, the mean value of the oxygen consumption at 37° C was $7{\cdot}07\,\mu\text{l.} \pm 0{\cdot}51$ (S.E. of 12)/mg final dry weight/hr, the gas volumes being reduced to 0° C and a pressure of one atmosphere. The determinations were made during the first 30 min after the muscles had been placed in the plastic chambers for manometric estimation. The mean water content was 78·2%, and the specific gravity was taken as 1·055 (Creese, 1954). The oxygen consumption may therefore be expressed as 0·027 c.c./c.c. wet tissue/min. The oxygen consumption recorded under these conditions is higher than that usually reported for rat diaphragm muscle, though the results of Pearson *et al.* (1949), who used phosphate saline, are comparable. They recorded a Q_{O_2} of 6·9. The values obtained on rats of 150–200 g are assumed to apply to the muscles from rats of 150–250 g which were used for electrical recording (cf. von Bertalanffy & Pirozynski, 1951).

The apparent diffusion coefficient may now be calculated. In eqn. (2) the slope is 0·0311 cm, a is 0·027 c.c./c.c./min, P is taken as one atmosphere and p, the vapour pressure of saline at 37° C, is 47 mm Hg. Then the Krogh diffusion coefficient of oxygen through diaphragm muscle at 37° C is $1{\cdot}4\times10^{-5}$ cm^2/min. The standard error of the mean, calculated according to Dahlberg (1948), is $0{\cdot}20\times10^{-5}$.

DISCUSSION

This investigation was started with the object of finding a method which could provide a convenient criterion for the occurrence of anoxia. The possibility that the oxygen supply is inadequate is often of pressing concern in the case of isolated mammalian tissues maintained at body temperature. Such preparations have a high oxygen consumption and oxygen must diffuse a considerable distance to reach the inner depths of the tissue. Frog muscle at room temperature consumes about 40 mm^3 oxygen/g/hr (Meyerhof & Schulz, 1927), which corresponds to about 0·0007 c.c./c.c./min. The oxygen consumption of diaphragm under the present conditions is about 0·027 c.c./c.c./min, which is nearly forty times greater.

Criteria of oxygenation previously adopted include the twitch tension, and the potassium and sodium content, which seems to be a sensitive indication of anoxia (e.g. Creese, 1954). An inadequate oxygen supply will result in a regional difference in resting potentials, for the outer portions of the tissue must receive more oxygen than the deeper parts. The present findings support the suggestion that regional differences in which the inner cells show reduced potentials may be used as a criterion of anoxia in isolated preparations.

Using the criteria both of the ionic content and of the micro-electrode method, the present authors have been able to obtain continuous adequate oxygenation only when the gas jet was arranged so that bubbles of oxygen were able to impinge directly on to the tissue. When the muscle was placed in a horizontal position and saline which had been aerated previously was passed over the tissue, it was found that the muscles showed a fall in potassium and a gain of sodium, and these findings were taken to indicate anoxia (Creese, 1954). The latter method of oxygenation would probably be adequate, however, for electrical studies on surface fibres.

The method used in the present work has the additional advantage that the records allow the estimation of the common physical dimensions of the stretched muscle, including thickness, fibre size and extrafibre space. The value of the inulin space of diaphragm muscle is 0·28, which is the same as the mean value found by micro-electrodes, and the space estimated by means of labelled sodium is also similar (Creese, 1954). In the case of frog sartorius muscle, however, there is a discrepancy. The fraction by volume of sartorius muscle into which inulin will penetrate is about 0·13 (Desmedt, 1953). The micro-electrode method gives 0·19. This last figure is in agreement with the results of Causey & Harris (1951), who obtained a value of 0·20 both with labelled phosphate ions and with labelled hexose phosphate. Harris & Burn (1949) similarly obtained a mean value of 0·18 in experiments with radioactive sodium. The discrepancy might be due to different and arbitrary degrees of wiping of muscles before estimations, or may be attributed to connective tissue. It is known that gaps between deflexions as recorded by the micro-electrode method may be produced by the presence of connective tissue, and this would be recorded as extrafibre space (Creese, Dillon, Marshall, Sabawala, Schneider, Taylor & Zinn, 1957).

Some attempt has been made to put on a semi-quantitative basis the results which relate the records of cell potentials to the oxygen concentration. Only steady states have been considered, and at least 3 hr were allowed before recording. The depth in the muscle at which depolarization below 15 mV occurs has been used as a bio-assay procedure to indicate the presence of a particular oxygen tension, which is itself unknown but which is likely to be below 7 mm Hg. This depth varies with the external oxygen concentration, and the relation of these variables has been obtained. It is assumed that oxygen consumption is independent of oxygen tension down to low values of the latter. In the case of frog muscle the work of Hill (1948) has shown that this relation is valid at 15–20° C down to 2 mm Hg or less. No reliable investigations are available in the case of mammalian muscle, but careful studies on other mammalian tissues at body temperature give support to this view. Thus Warren (1942), who worked with cell suspensions from bone marrow, and Elliott & Henry (1946), who studied suspensions of brain tissue, have shown that oxygen consumption is independent of oxygen tension down to at least 4–6 mm Hg, providing the results are free from distortion produced by diffusion and solubility factors affecting the amount of oxygen in the saline solution. The present experimental findings though variable are consistent with these assumptions, and there seems therefore no reason to invoke more cumbersome mathematical treatment.

The interpretation of the quantitative results is complicated by the possibility that lactic acid is being produced by the anaerobic muscle in sufficient quantity to affect markedly the consumption of oxygen. Lactic acid is unlikely to be produced by cells with normal potentials nor by cells with very low potentials which are probably functionless; but cells which are somewhat depolarized probably produce lactate or other 'debt metabolites'. The zone of partial depolarization does not extend over many cell layers. Hill (1948) has shown that under similar conditions the parabolic relation between oxygen tension and distance is maintained down to very low tensions in frog muscle, though the oxygen consumption was raised in the anoxic zone. Hannon & Cook (1956) have detected a transient increase in oxygen consumption after short exposure to nitrogen in the case of mouse diaphragm. If the figure used for oxygen consumption in eqn. (2) is too small then the calculated value of the diffusion coefficient will be somewhat smaller than the true value, and this may account for the rather low figure actually obtained. The altered potassium content of cells in the zone of depolarization might also be expected to alter the metabolic rate to some extent. It is known that a high external potassium concentration increases the oxygen consumption of frog muscle (Keynes & Maisel, 1954), and in the present experiments the external saline was therefore changed frequently.

The value of the Krogh diffusion coefficient as calculated from the slope of the regression line is $1{\cdot}4\times10^{-5}$ cm^2/min at 37° C. There is no direct measurement made on mammalian muscle with which the results may be compared. Writers on oxygen diffusion for the last forty years have used the value of the mixed diffusion coefficient originally published by Krogh in 1919 (*a*), which was based on measurement made on abdominal muscle of the frog, at 20° C. The figure of $1{\cdot}64\times10^{-5}$ cm^2/min at 37° C was calculated by Krogh (1919*b*) by extrapolation of results obtained at lower temperatures, and the value found in the present study is some 15% lower than that used by Krogh. This difference is not significant since the S.E. is $\pm0{\cdot}20\times10^{-5}$. Hill (1928) and others have used the figure $1{\cdot}7\times10^{-5}$ cm^2/min at 37° C. Considering the limitations of the methods employed, the result in the present experiments is not unsatisfactory. It is difficult to devise a method of measuring the diffusion coefficient directly in mammalian muscle while maintaining the tissue properly supplied with oxygen, and such information as is known about oxygen transport in mammalian tissues has been obtained largely by indirect means, as in the present study.

SUMMARY

1. A method of exploring muscle is described in which a motor is used to push a micro-electrode slowly through the tissue. Resting potentials of fibres which are encountered are registered by a pen recorder.

2. The method may be used to measure certain physical dimensions of the tissue. For stretched diaphragm muscle of the rat the extrafibre space was 0·28 of the total volume. The value for the sartorius muscle of the frog was 0·19. The mean fibre diameter of diaphragm muscles from rats of 200–300 g was 32μ, after corrections for systematic bias during measurements.

3. In the presence of a constant carbon dioxide concentration, reduction of the oxygen content in the gas mixture used to aerate the tissue results in depolarization in the centre of the muscle, while the superficial fibres are normal. These findings are characteristic, and may be used as a criterion of anoxia.

4. The oxygen consumption, obtained by manometric methods in the presence of 2% carbon dioxide, was 0·027 c.c. (s.t.p.)/c.c. wet tissue/min at 37° C during the first 30 min after diaphragm muscles from rats of 150–200 g were placed in the chambers.

5. The method was also used to obtain information regarding oxygen requirements and oxygen transport in mammalian muscle. The Krogh diffusion coefficient for oxygen at 37° C in diaphragm was estimated as $1{\cdot}4 \times 10^{-5}$ cm^2/min.

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APPENDIX, BY R. CREESE AND D. J. JENDEN

Expressions are required which give the mean thickness and standard deviation of muscle fibres from values obtained by random impaling by micro-electrodes in which chords are measured instead of diameters.

The cells are treated as cylinders and their radii are assumed to be distributed in a lognormal manner. There is some evidence for this latter assumption (Creese, Neil & Stephenson, 1956; Creese, D'Silva & Hashish, 1955). It is also considered that the probability of encountering a cell of given radius is proportional both to its distribution frequency and also to its radius.

Consider first a single fibre of radius r. It is convenient to compare r with the semichord l, where l is half the measured thickness. The frequency with which a given class of lengths is encountered is proportional to the class interval along the X-axis corresponding to it. Then $f(l)$, the probability density function, may be constructed as follows.

The conditions are

$$\int_0^r f(l)\,\mathrm{d}l=1 \tag{4}$$

and

$$\mathrm{d}N=f(l)\,\mathrm{d}l, \tag{5}$$

where $\mathrm{d}N$ gives the number of semichords encountered in the interval $\mathrm{d}x$, and $\mathrm{d}N$ is proportional to $\mathrm{d}x$. Now x is given by $(r^2-l^2)^{\frac{1}{2}}$ and $\mathrm{d}x/\mathrm{d}l$ is $-l/(r^2-l^2)^{\frac{1}{2}}$, and to satisfy eqn. (4) we have

$$f(l)=l/\{r(r^2-l^2)^{\frac{1}{2}}\}. \tag{6}$$

The first moment α gives the mean semichord:

$$\int_0^r lf(l)\,\mathrm{d}l=\pi r/4. \tag{7}$$

The second moment β gives the mean squared semichord:

$$\int_0^r l^2f(l)\,\mathrm{d}l=2r^2/3. \tag{8}$$

The third moment γ gives the mean cubed semichord:

$$\int_0^r l^3f(l)\,\mathrm{d}l=3\pi r^3/16. \tag{9}$$

The skewness statistic g, given by (3rd moment about mean):(standard deviation cubed), is $-1{\cdot}15$, so that the distribution of semichords (and of chords) in a single cell has a markedly negative skew.

Now consider the distribution of the radii. If this is lognormal then the logarithms of the radii have a Gaussian distribution such that

$$f(\ln r)=(2\pi\lambda^2)^{-\frac{1}{2}}\exp\left[-\{\ln(r/r_m)\}^2/2\lambda^2\right], \tag{10}$$

where r_m is the mode and also the geometric mean, and λ is the standard deviation of $\ln r$. Hence if a similar condition to that stated in eqn. (4) is followed, we have

$$f(r)=r^{-1}(2\pi\lambda^2)^{-\frac{1}{2}}\exp\left[-\{\ln(r/r_m)\}^2/2\lambda^2\right]. \tag{11}$$

It can be shown (see Finney, 1941) that the nth moment is given by

$$E(r^n)=\int_0^\infty r^nf(r)\,\mathrm{d}r=r_m^n\exp(n^2\lambda^2/2). \tag{12}$$

In particular the mean $\bar{r}$ and the S.D. σ are given by

$$\bar{r}=\int_0^\infty rf(r)\,\mathrm{d}r=r_m\exp(\lambda^2/2) \tag{13}$$

and

$$\sigma^2=\int_0^\infty r^2f(r)\,\mathrm{d}r-(\bar{r})^2=(\bar{r})^2\{(\exp\lambda^2)-1\}. \tag{14}$$

These results have been stated by Finney (1941).

The parameters of the population of semichords which is found experimentally is now deduced. The probability of encountering a cell of given radius is proportional to $rf(r)$. The distribution of means α is also proportional to $rf(r)$, and $f(\alpha)$ follows a similar condition to that of eqn. (4). The proportionality factor is found as follows. By eqn. (7), $\mathrm{d}\alpha$ equals $(\pi/4)\,\mathrm{d}r$. Now

$$\int_0^\infty rf(r)\,\mathrm{d}\alpha=(\pi/4)\int_0^\infty rf(r)\,\mathrm{d}r=(\pi/4)r_m\exp(\lambda^2/2). \tag{15}$$

Hence

$$f(\alpha)=(4/\pi)\{r_m\exp(\lambda^2/2)\}^{-1}\,rf(r). \tag{16}$$

Then l_a, which is the grand mean of the observed population of semichords, is given by the first moment of α where

$$l_a=\int_0^\infty \alpha f(\alpha)\,\mathrm{d}\alpha=(\pi/4)r_m\exp(3\lambda^2/2). \tag{17}$$

The distribution of β is also proportional to $rf(r)$, and in a similar manner it follows that

$$f(\beta) = (3/4)\,\{r_m^2 \exp(2\lambda^2)\}^{-1}\, rf(r). \tag{18}$$

The mean of the β values is given by the first moment of β, and hence σ_a^2, the variance of the observed population, is

$$\int_0^\infty \beta f(\beta)\,\mathrm{d}\beta - (\bar{l}_a)^2 = (2/3)\, r_m^2 \exp(6\lambda^2) - (\bar{l}_a)^2. \tag{19}$$

The distribution of the mean cubed semichord γ is also proportional to $rf(r)$, and similarly

$$f(\gamma) = (16/9\pi)\,\{r_m^3 \exp(9\lambda^2/2)\}^{-1}\, rf(r). \tag{20}$$

The mean cubed semichord of the observed population is given by the first moment of γ, and hence the mean cubed deviation G of the observed population is

$$\int_0^\infty \gamma f(\gamma)\,\mathrm{d}\gamma - (\bar{l}_a)^3 - 3\bar{l}_a\sigma_a^2 = (3\pi/16)\, r_m^3 \exp(27\lambda^2/2) - (\bar{l}_a)^3 - 3\bar{l}_a\sigma_a^2. \tag{21}$$

Since both $\bar{l}_a$ and σ_a have been determined experimentally, it is of value to express r_m and λ in terms of them. Let the term $\{(\bar{l}_a)^2 + \sigma_a^2\}/(\bar{l}_a)^2$ be designated by t. Then from eqn. (17) and eqn. (19) we have

$$\exp(3\lambda^2) = 3\pi^2 t/32 \tag{22}$$

and

$$r_m^2 = 512\,(\bar{l}_a)^2/(3\pi^4 t), \tag{23}$$

also

$$G = 81\,\pi^4 t^3 (\bar{l}_a)^3/8192 - (\bar{l}_a)^3 - 3\bar{l}_a\,\sigma_a^2. \tag{24}$$

The experimental value of the mean chord thickness, which is $2\bar{l}_a$ in the above nomenclature, is $26{\cdot}7\mu$. The corrected mean diameter, which is $2\bar{r}$, is $31{\cdot}6\mu$. These fibres are from stretched diaphragm muscles from rats of 200–300 g.

Creese (1954) obtained a value of about 20μ by histological methods in the case of diaphragm muscles from rats of 120 g.

The observed S.D. of chord thickness is $\pm 15{\cdot}7\,\mu$, and this corresponds to $2\sigma_a$. The corrected value, given by 2σ, is $\pm 8{\cdot}7\mu$. The value of λ is $0{\cdot}27$ $\log_e$ units, or $0{\cdot}12$ $\log_{10}$ units. This may be compared with the value of $0{\cdot}076$ $\log_{10}$ units found by histological methods (Creese *et al.* 1956). The greater scatter in the present study may be attributed to the larger range in the weights of the rats.

The experimental value of g, the skewness statistic, is $+1{\cdot}1$. The value computed with the aid of eqn. (24) is $+1{\cdot}5$.

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USE OF THE STRAIN GAUGE PLETHYSMOGRAPH IN ASSESSING THE EFFECT OF CERTAIN DRUGS ON THE BLOOD FLOW THROUGH THE SKIN AND MUSCLE OF THE HUMAN FOREARM

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The values obtained for forearm blood flow by a mercury-in-rubber strain gauge placed midway between elbow and wrist have been shown to be similar to those recorded by a water-filled venous occlusion plethysmograph (Clarke & Hellon, 1957). The comparison was made over a wide range of flows produced by direct heating. Since a most useful application of the strain gauge technique would be in conditions of rapid circulatory change, a further comparison between the two methods was considered desirable. Forearm blood flow has therefore been measured by both methods simultaneously during the administration of certain drugs with known circulatory effects.

Forearm blood flow measured by the strain gauge has been found to vary with the level at which the gauge is mounted on the arm, and it was suggested that this difference might be related to the varying proportions of skin and muscle along the forearm (Clarke & Hellon, 1957). A second aim of these experiments was therefore to determine whether peripheral responses to these drugs varied at different levels along the forearm, and could be related to a differential action of the drugs on skin and muscle. The three drugs given in this study, adrenaline, noradrenaline and pitressin, were therefore chosen from among those previously shown to have different actions on the vessels of skin and muscle.

METHODS

The subjects, eight healthy young men and one woman, lay recumbent with both forearms about 10 cm below heart level. The strain gauges used were essentially as described by Whitney (1953), applied to the arm at a tension of 20 g. Two gauges were mounted on the right arm in standardized positions, 6 cm above and 6 cm below the mid point between olecranon and styloid process of the ulna. The arm was then immersed to above the elbow in a stirred water-bath maintained at 34° C.

The venous occlusion plethysmograph was as described by Barcroft & Swan (1953), filled with water at 34° C, and placed on the left forearm. Cuffs were inflated at the wrist to 200 mm Hg 1 min before starting recording blood flow. Venous occlusion cuffs, placed immediately above the elbow, were inflated from a common source to 65 mm Hg. Each occlusion was maintained for 7 sec and repeated four times each minute.

A continuous infusion of normal saline (NaCl 0·9% w/v) was maintained into an antecubital vein of the left arm. Blood flow was measured for 5 min during saline infusion, for 10 min with the drug added to the saline and for 5 min with saline again. Dosages were as follows:

(1) L-Adrenaline hydrogen tartrate (British Drug Houses) at 10 μg/min.

(2) L-Noradrenaline tartrate (Levophed, Bayer Products) at 10 μg/min.

(3) Vasopressin (Pitressin, Parke, Davis) at 0·17 u./min.

Room temperature varied from 20 to 23° C in different experiments.

RESULTS

Adrenaline

In Fig. 1 is plotted the mean blood flow of six subjects, as recorded by both gauges and the plethysmograph. The response was qualitatively similar in both arms, but, compared with the plethysmograph, the upper and lower gauges showed larger and smaller blood flows respectively throughout the infusion. Table 1 compares the mean flow in each individual during the last 4 min of infusion with that in the 4 min immediately preceding the infusion. In most

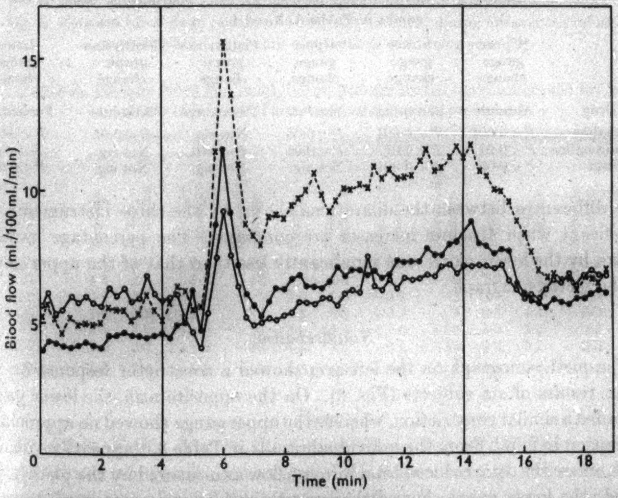

Fig. 1. Forearm blood flows (means of 6 subjects) measured by the plethysmograph (●), and upper (×) and lower (○) strain gauges during intravenous infusion of adrenaline (10 μg/min). The duration of the infusion is shown by the vertical lines.

subjects the upper gauge showed the largest increase in flow (both absolute and percentage), while the lower gauge showed the smallest change, or in some cases even a decrease. The plethysmograph gave an intermediate response in each subject.

An analysis of variance has been carried out on the absolute and percentage changes in flow shown in Table 1. The probabilities obtained when the differences between the three instruments are considered, are given in Table 2.

TABLE 1. Forearm blood flow in ml./100 ml./min during the 4 min before, and the last 4 min of infusion of adrenaline (10 μg/min for 10 min)

	Plethysmograph				Upper gauge				Lower gauge			
Subject	Resting	Drug infusion	Absolute change	Percentage change	Resting	Drug infusion	Absolute change	Percentage change	Resting	Drug infusion	Absolute change	Percentage change
R.C.	3·6	8·8	+5·2	+144	5·4	11·9	+6·5	+119	6·5	5·5	−1·0	−15
M.W.	7·4	9·6	+2·2	+30	6·8	13·6	+6·8	+100	6·4	8·2	+1·8	+28
J.E.	3·6	9·0	+5·4	+150	5·3	15·8	+10·5	+198	4·5	8·7	+4·2	+93
D.P.	2·8	5·8	+3·0	+107	5·4	11·7	+6·5	+117	6·3	6·9	+0·6	+10
P.H.	4·2	5·3	+1·1	+26	4·8	5·8	+1·0	+21	5·1	4·3	−0·8	−16
M.D.	4·2	5·5	+1·3	+31	4·1	6·7	+2·6	+63	7·4	7·1	−0·3	−4
Mean	4·3	7·3	+3·0	+81	5·3	10·9	+5·6	+106	6·0	6·8	+0·8	+16

TABLE 2. Significance of the differences between the three instruments, based on the results in Tables 1, 3 and 4

	Upper gauge change > Lower gauge change		Upper gauge change > Plethysmograph change		Plethysmograph change > Lower gauge change	
Drug	Absolute	Percentage	Absolute	Percentage	Absolute	Percentage
Adrenaline	$P<0{\cdot}001$	$P<0{\cdot}001$	$P<0{\cdot}05$	Not sig.	$P<0{\cdot}05$	$P<0{\cdot}01$
Noradrenaline	$P<0{\cdot}01$	$P<0{\cdot}01$	$P<0{\cdot}05$	$P<0{\cdot}01$	Not sig.	Not sig.
Pitressin	$P<0{\cdot}05$	$P<0{\cdot}001$	Not sig.	Not sig.	Not sig.	$P<0{\cdot}01$

The differences between the dilatations shown by the three instruments are significant when absolute increases are compared. The percentage increase shown by the lower gauge was significantly less than that of the upper gauge or the plethysmograph.

Noradrenaline

The plethysmograph on the left arm showed a constrictor response on the mean results of six subjects (Fig. 2). On the opposite arm, the lower gauge recorded a similar constriction, whereas the upper gauge showed no appreciable alteration in flow. From the individual results in Table 3 it appears in general that, where the drug reduced total forearm flow as measured by the plethysmograph, the lower gauge showed the largest absolute and percentage decrease of the three instruments; conversely where total forearm flow increased, the lower gauge showed the smallest increase.

Analysis of variance (Table 2) reveals that the change, both absolute and percentage, in the upper gauge flow was significantly less than that shown by the plethysmograph or the lower gauge.

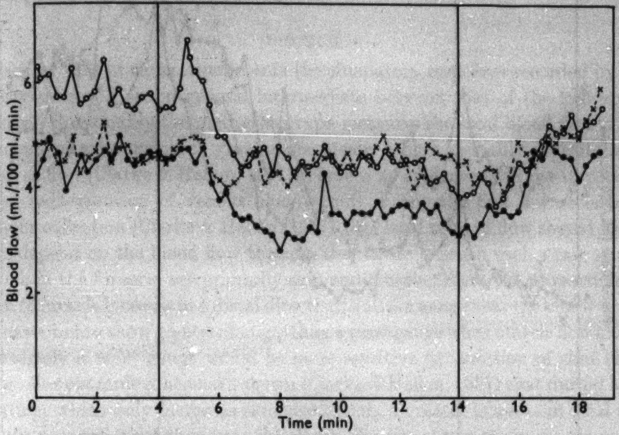

Fig. 2. Forearm blood flows (means of 6 subjects) measured during intravenous infusion of noradrenaline (10 μg/min). Conventions and timing as in Fig. 1.

Table 3. Forearm blood flow in ml./100 ml./min during the 4 min before, and the last 4 min of infusion of noradrenaline (10 μg/min for 10 min)

Subject	Plethysmograph				Upper gauge				Lower gauge			
	Resting	Drug infusion	Absolute change	Percentage change	Resting	Drug infusion	Absolute change	Percentage change	Resting	Drug infusion	Absolute change	Percentage change
R.C.	3·1	2·7	−0·4	−13	3·9	4·1	+0·2	+5	7·2	7·4	+0·2	+3
J.E.	4·7	2·5	−2·2	−47	6·4	4·5	−1·9	−30	7·2	3·2	−4·0	−55
D.P.	3·1	3·1	+0·0	+0	5·0	4·9	−0·1	−2	5·7	5·1	−0·6	−10
P.H.	7·6	5·2	−2·4	−32	5·3	5·8	+0·5	+9	6·3	4·1	−2·2	−35
J.G.	4·1	4·9	+0·8	+20	3·0	4·6	+1·6	+53	3·0	3·5	+0·5	+17
M.D.	4·5	2·7	−1·8	−40	4·3	3·7	−0·6	−14	5·6	3·1	−2·5	−45
Mean	4·5	3·5	−1·0	−19	4·6	4·6	+0·0	+4	5·8	4·4	−1·4	−21

Pitressin

The effect of this drug on the forearm blood flow of different individuals (Table 4) was more variable than that of the other two drugs. The mean results in Fig. 3 indicate a graded dilator response from the lower gauge, through the plethysmograph, to the upper gauge. There was a slight reduction in flow during the first few minutes, plainest with the lower gauge, and after 5–8 min

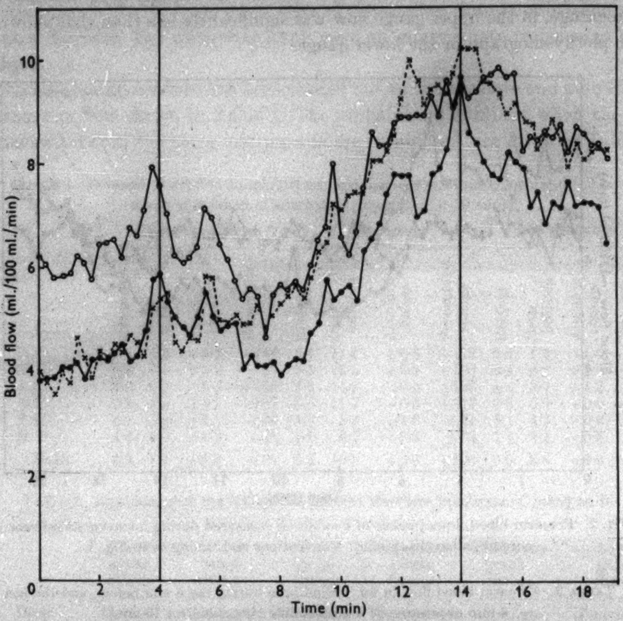

Fig. 3. Forearm blood flows (means of 6 subjects) measured during intravenous infusion of Pitressin (0·17 unit/min). Conventions and timing as in Fig. 1.

TABLE 4. Forearm blood flow in ml./100 ml./min during the 4 min before, and the last 2 min of infusion of Pitressin (0·17 u./min for 10 min)

Subject	Plethysmograph				Upper gauge				Lower gauge			
	Resting	Drug infusion	Absolute change	Percentage change	Resting	Drug infusion	Absolute change	Percentage change	Resting	Drug infusion	Absolute change	Percentage change
R.C.	2·7	5·3	+2·6	+96	4·8	11·9	+7·1	+148	7·9	12·1	+4·2	+53
J.E.	4·0	7·8	+3·8	+95	5·5	12·7	+7·2	+131	6·6	10·4	+3·8	+58
P.H.	5·0	7·3	+2·3	+46	2·2	3·6	+1·4	+64	4·0	5·3	+1·3	+33
M.D.	3·8	4·0	+0·2	+5	3·3	5·7	+2·4	+73	5·2	4·0	−1·2	−23
P.S.	3·5	8·6	+5·1	+146	4·4	12·1	+7·7	+175	7·4	11·2	+3·8	+51
J.H.	6·6	14·9	+8·3	+126	5·6	11·0	+5·4	+96	7·0	11·2	+4·2	+60
Mean	4·3	8·0	+3·7	+86	4·3	9·5	+5·2	+115	6·4	9·0	+2·6	+39

an abrupt rise occurred continuing to the end of the infusion. The mean flow during the last 2 min of infusion is compared with resting levels in Table 4, but because of the wide scatter analysis of variance (Table 2) shows that only the difference between the gauges is significant.

DISCUSSION

It is clear that in these experiments the circulatory responses recorded by the plethysmograph are in general intermediate between that of the two strain gauges. Presumably the plethysmograph measures the total blood flow in the segment of arm enclosed, whereas the strain gauges probably indicate local rates of flow (Clarke & Hellon, 1957). There is no evidence that any important axial redistribution of venous blood occurs during the first few seconds of venous collection (Clarke & Hellon, 1957). The local rates of flow should therefore depend on the blood flow through the tissues beneath each strain gauge, which in the forearm are primarily skin and muscle. Since the proportions of skin to muscle increase in a distal direction, a strain gauge near the elbow would be expected to show greater changes than a wrist gauge when muscle flow alters; conversely a wrist gauge would be more sensitive to variation of skin blood flow. For example it has been shown (Clarke & Hellon, 1957) that during body heating, which only increases skin circulation, a greater increase in local flow occurs near the wrist than near the elbow. The use of two strain gauges on the forearm should therefore indicate, at least qualitatively, whether a given procedure is changing peripheral blood flow in the skin or muscle vessels. The applications of this principle to the present experiments may now be undertaken. In the considerations which follow, it has been assumed that there is no appreciable variation in blood flow per unit volume of skin or muscle along the forearm. There is no evidence available to show whether this is correct or not.

The transient and sustained rises in forearm blood flow during intravenous infusions of adrenaline were first described by Allen, Barcroft & Edholm (1946). They showed that the transient rise was the result of a direct action on the vessels, and the subsequent work of Duff & Swan (1951) and Whelan (1952) suggested that the sustained dilatation was due to a dilator substance liberated centrally, which acted on limbs with intact or recently cut sympathetic nerves. The blood flow through the hand falls at once and only returns to normal after the infusion ceases (Swan, 1949). This evidence, together with the general pallor of the skin during infusions of adrenaline, suggests that skin vessels throughout the body are constricted. If the skin vessels of the forearm were made to constrict and the muscle vessels were dilating, the latter effect would be expected to be more evident in a gauge placed near the elbow and the former effect in a gauge near the wrist; the volume plethysmograph, which samples a longer segment of the forearm, would show an intermediate effect. The results obtained with the two strain gauges are therefore compatible with the view that forearm skin vessels, like those of the hand, are constricted by adrenaline.

The action of noradrenaline is more difficult to analyse since various workers have reported different effects. Duncanson, Stewart & Edholm (1949) and Barnett, Blacket, Depoorter, Sanderson & Wilson (1950) found a decrease in forearm flow; Barcroft & Konzett (1949) and Whelan & Young (1953), however, often found a slight transient or sustained increase. The dilatation seen in the forearm with intravenous noradrenaline is never seen in the calf or hand (Swan, 1949; de Largy, Greenfield, McCorry & Whelan, 1950). One might therefore conclude that noradrenaline was constrictor to skin vessels but variable in its effects on those of muscle. It now appears (Barcroft, Gaskell, Shepherd & Whelan, 1954) that there is a direct constrictor action on muscle vessels combined with a dilator action which is mediated by sympathetic nerves. The present results from the two strain gauges show a marked constriction with the lower gauge and little change with the upper gauge. This suggests that noradrenaline is exerting a constrictor action on forearm skin and that its indirect dilator action is predominating on the muscle vessels. Since in these circumstances the plethysmograph shows only a reduction in total forearm flow, it may be concluded that, with dosage used, the constrictor action on the skin more than counterbalances the dilator action in the muscle.

The effect of an intravenous infusion of Pitressin on upper limb blood flow was described by Kitchin (1957): using the same dosage, the response found here with the plethysmograph was similar. Kitchin recorded a variable amount of reduction in hand flow and a delayed increase in forearm flow. He concluded therefore that this drug was a constrictor of skin vessels and a dilator of muscle vessels. The results with the two strain gauges are again consonant with this conclusion, and, since the dilator action is quite marked even with the lower gauge, indicate that the constrictor element at this dosage is less pronounced than with adrenaline.

The foregoing type of analysis of the changes in skin and muscle blood flow as indicated by the two strain gauges has obvious limitations, and could not be applied if vasodilatation or vasoconstriction occurred in both tissues simultaneously. This difficulty could be overcome and the analysis made quantitative by an algebraic amalgamation of any pair of results from two gauges; a direct and separate measure of skin and muscle blood flow would then be theoretically possible. The derivation of two equations for this analysis is given in the Appendix. For substitution in these equations, blood flow through bone, tendon, fat and fascia has been assumed to be of the order of 1 ml./100 ml./min (Edholm, Howarth & McMichael, 1945; Cooper, Edholm & Mottram, 1955), and to vary little with the drugs used.

This method requires a knowledge of the composition of the forearm beneath each strain gauge, which cannot be determined for any given subject. However, using figures calculated from published cross-sections of the forearm (Morton, Truex & Kellner, 1941), the two equations give in a group of subjects values for skin and muscle flows which are not unreasonable. For example, with the mean values in Table 1, resting figures for skin and muscle flow are 45 ml./100 ml. skin/min and 3 ml./100 ml. muscle/min respectively. During the last 4 min of the adrenaline infusion, the corresponding values are 4 and 14·6 ml. Owing to the uncertainty about the composition of the forearms of our subjects, this method of analysis has not been applied to the other results in this paper. Until some way is found to overcome this difficulty, it appears justifiable to apply this technique of distinguishing between skin and muscle blood flow only semi-quantitatively. It does, however, provide an alternative method to that of Cooper *et al.* (1955), using adrenaline iontophoresis, which is only suitable for steady conditions of blood flow.

SUMMARY

1. Changes in forearm circulation have been followed by a venous occlusion plethysmograph mounted on one arm and two mercury-in-rubber strain gauge plethysmographs 12 cm apart on the other, during the intravenous infusion of adrenaline, noradrenaline and Pitressin.

2. The change shown by the plethysmograph was intermediate between that shown by the two strain gauges for all three drugs.

3. It is suggested that the local blood-flow figures given by the strain gauges are related to the proportions of skin and muscle in the two positions on the forearm. The results from the gauges are in agreement with the known effects of these drugs on skin and muscle blood flow.

4. A method of analysis whereby the results from the two gauges could give quantitative estimates of skin and muscle blood flow is outlined.

We should like to thank Professor Henry Barcroft, F.R.S., for his advice and encouragement throughout this work, Miss Rosemary Morton for statistical advice, Miss Robina Palmer for technical assistance, and the subjects for their willing co-operation. Certain expenses were defrayed by a grant from the Endowment Fund of St Thomas's Hospital.

APPENDIX

If the conclusion reached by Clarke & Hellon (1957) is accepted, namely that there is no axial redistribution of blood along the forearm during venous collection, then at any given level of the forearm,

$$100F = Ss + Mm + Bb, \tag{1}$$

where F is forearm blood flow in ml./100 ml. forearm/min, s, m and b are the percentages of skin, muscle and bone, etc., at that level and S, M and B are the blood flows in ml./100 ml. tissue/min through these three tissues. Using U and L as suffixes for the levels of forearm 6 cm above and

6 cm below the mid point, and assuming that the blood flow through a particular tissue does not vary throughout the forearm, equation (1) may be rewritten for the two positions:

$$S=\frac{100F_U-Mm_U-Bb_U}{s_U} \qquad (2)$$

and

$$S=\frac{100F_L-Mm_L-Bb_L}{s_L}. \qquad (3)$$

Subtracting (3) from (2)

$$100F_Us_L-100F_Ls_U+Mm_Ls_U-Mm_Us_L+Bb_Ls_U-Bb_Us_L=0;$$

therefore

$$M=\frac{100\,(F_Us_L-F_Ls_U)+B\,(b_Ls_U-b_Us_L)}{m_Us_L-m_Ls_U}.$$

Similarly it may be shown that

$$S=\frac{100\,(F_Um_L-F_Lm_U)+B(b_Lm_U-b_Um_L)}{s_Um_L-s_Lm_U}.$$

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SOME OBSERVATIONS CONCERNING THE MODE OF ACTION OF ACETYLCHOLINE IN ISOLATED RABBIT ATRIA

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Previous studies of the effects of vagal stimulation or of acetylcholine upon conduction velocity in mammalian atrial muscle have not always led to the same conclusions. Lewis, Drury & Bulger (1920) observed in the dog no change in conduction velocity after vagal stimulation, but Brooks, Hoffman, Suckling & Orias (1955) reported that conduction velocity was increased. Burgen & Terroux (1953*b*) observed in the cat a decrease in conduction velocity in strips of auricle exposed to ACh and carbamylcholine, but Ramos & Rosenblueth (1947) found an increase, which they attributed to the shortening of the duration of the action potential. A preliminary investigation in rabbit atria (Vaughan Williams, 1954) had shown that ACh sometimes had a diphasic effect on conduction velocity, the usual increase in velocity occasionally being preceded or interrupted by a temporary phase of reduced velocity. The present paper is largely devoted to an attempt to explain these effects in terms of the changes produced by ACh in the various parameters of the action potential recorded intracellularly.

METHODS

Isolated rabbit atria were suspended horizontally in apparatus which has already been described (Vaughan Williams, 1955; Marshall & Vaughan Williams, 1956); the temperature was 30–31° C, except where otherwise stated. Conduction velocity was measured with bipolar platinum electrodes. Intracellular records were obtained with conventional methods, the input valve being an ME 1400 mounted on the micromanipulator. The input time constant was 40 μsec, measured by injecting a square wave into the bath earth line, with an 11 MΩ micropipette just touching the surface of the solution (capacity = 4 pF). In order to measure the rate of rise of the upstroke of the intracellular action potential, a high speed of the oscilloscope sweep was necessary, and the following procedure was adopted to obtain photographs on stationary film of the required part of the trace, when the auricles were beating spontaneously. An external electrode was placed on the pace-maker, and the action potential from this was used to trigger the sweep. The microelectrode was inserted some distance away, the conduction time from external to internal electrode providing the necessary delay. A pulse was then taken from the end of the sweep and used to operate the camera movement. As soon as the film was again stationary another pulse operated a shutter exposing a clock face, to give the time of exposure accurate to within 0·1 sec. Changes in the rate of the pace-maker could thus be calculated from beat to beat, and the camera simply followed the atrial rhythm, thus avoiding much waste of film when the pace-maker was arrested for long periods.

Solution flowed continuously past the preparation at a rate which ensured complete removal within 15 min of a dye introduced as a test substance. Acetylcholine was added either to the bath in a single amount in a small volume, so that the concentration started high and then fell to zero within a few minutes; or to the supply reservoir so that the auricle was exposed to a more constant concentration. In the earlier experiments contractions were recorded by a lever writing on a smoked drum, but in the later experiments, to obtain absolute accuracy of synchronization, the contractions were measured by an RCA 5734 transducer, whose output was photographed with the corresponding action potentials on the same film.

RESULTS

The effects on conduction velocity of various concentrations of ACh were observed in 110 experiments on twenty-nine isolated rabbit atria. In a few experiments the higher concentrations of ACh induced irregularities of electrical activity which temporarily invalidated measurements of conduction velocity. These irregularities could be classified into three categories.

(*a*) A change in the site of the pace-maker. One of the bipolar electrodes was placed on or near the pace-maker, so that a change in the site of origin of electrical activity was shown by a change in the shape of the first recorded action potential. A change of pace-maker was rare, occurring in only two auricles.

(*b*) A 'saltatory' change in the pathway of conduction. In nine experiments in five auricles it was observed that the second action potential, without changing its shape, suddenly became much closer to the first (Fig. 1*B*). This change occurred in the interval between one beat and the next, and was so large that it was concluded that it was not due to an increase in conduction velocity, but to the impulse jumping a whole group of fibres instead of being conducted along them. This saltatory effect was never observed with concentrations of ACh lower than 10^{-6}. In most auricles it was never seen, even with concentrations sufficient to stop the pace-maker for many seconds.

(*c*) Development of an ectopic pace-maker. In two experiments, although the regular frequency and shape of the first action potential was unaltered, indicating that there was no change in the original pace-maker, the second action potential exhibited an independent frequency, implying that the part of the atrium under the second electrode had broken away from the original pace-maker and was following an ectopic focus. This phenomenon is illustrated in Fig. 1*C*. Here the second action potential, whose normal shape was that shown in Fig. 1*C*, 3, became inverted when it no longer followed the original rate (Fig. 1*C*, 1, 2, 4) implying that the ectopic focus was on the opposite side of the electrode from the true pace-maker.

In practice these changes in pace-maker or pathway, which could have invalidated conclusions about changes in conduction velocity, were usually not difficult to recognize owing to the suddenness of their occurrence and the concomitant changes in shape of the action potential (cf. Burgen & Terroux, 1953*b*). Moreover, true changes in conduction velocity still occurred even when calculated from a new pace-maker or via a different pathway. The usual effect of ACh was to produce a change in conduction velocity which developed gradually from beat to beat and followed a characteristic time course during the inhibition and recovery of activity after exposure of the atria to the drug. In Fig. 1*A*, the changes in conduction velocity observed when 15 μg ACh (10^{-6} peak concentration) was added to the bath have been plotted, together with the changes occurring simultaneously in the frequency of the beat. There was no change in the site of the pace-maker, but during the phase of recovery there was a sudden shift in the conduction pathway (Fig. 1*B*) for a brief period, shown as a gap in the curves in fig. 1*A*. The second action potential still followed the pace-maker in a regular manner, but at a much shorter interval, illustrating a brief phase of saltatory conduction.

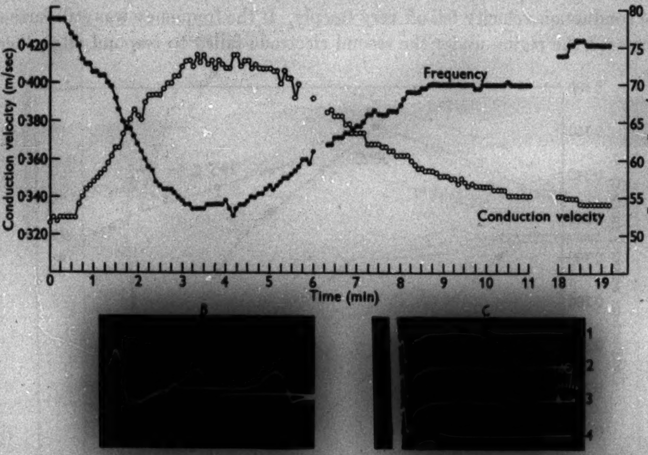

Fig. 1. *A*: simultaneous record of the effect of ACh on conduction velocity (○) and frequency of contractions (●). *B*: saltatory conduction without change of site of pace-maker. *C*: region under second electrode no longer following original pace-maker in 1, 2 and 4.

Increases in conduction velocity were observed in every experiment in which the auricles were exposed to sufficient ACh to reduce the contractions by half or more. At low concentrations of ACh, less than 10^{-6}, the increase in conduction velocity was frequently large, but in some experiments low concentrations produced changes only on the borderline of significance.

Effect of frequency

When atria were driven electrically up to frequencies of 300/sec, it was found that there was a linear inverse relation between frequency and conduction velocity. In Fig. 2 are shown the results of experiments in three preparations illustrating this point at three different temperatures. At higher frequencies the conduction velocity fell off very steeply. If the frequency was still further increased the region under the second electrode failed to respond altogether.

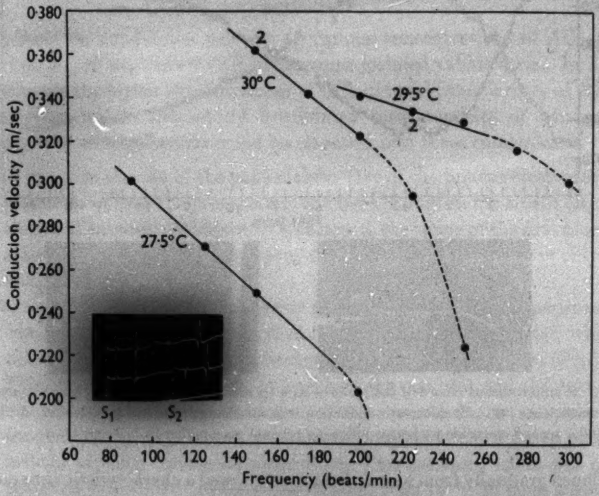

Fig. 2. Relation between conduction velocity and frequency of stimulus at different temperatures, in three auricles. At the points marked 2 the measurements were repeated at the end of the run and found to be the same. Inset: experiment showing that conduction velocity was reduced when the atria responded to a stimulus falling in the relative refractory period. S_1 and S_2, stimulus artifacts. Each stimulus artifact was followed by action potentials recorded from external electrodes on the right and left atria. The atria were driven at a constant rate (S_1) and a second stimulus was administered outside the relative refractory period (lower trace), and then within the relative refractory period (upper trace), whereupon the interval between the action potentials was prolonged.

The very much steeper reduction in conduction velocity (Fig. 2) at higher frequencies can be explained by the encroachment of the stimulus on the refractory period, since conduction velocity is much slower when the stimulus falls in the refractory period (Fig. 2, inset).

The linear relation between frequency and conduction velocity observed in the driven preparation suggested that the increases in conduction velocity seen after ACh in spontaneously beating auricles might be a consequence of the simultaneous slowing of the rate. The slowing cannot, however, entirely account for the changes in conduction velocity for three reasons. (1) When the changes in conduction velocity and rate were followed from beat to beat it was found that they were not correlated in time. (2) Sometimes there was an early phase of *decreased* conduction velocity (see below) when the rate was already slower.

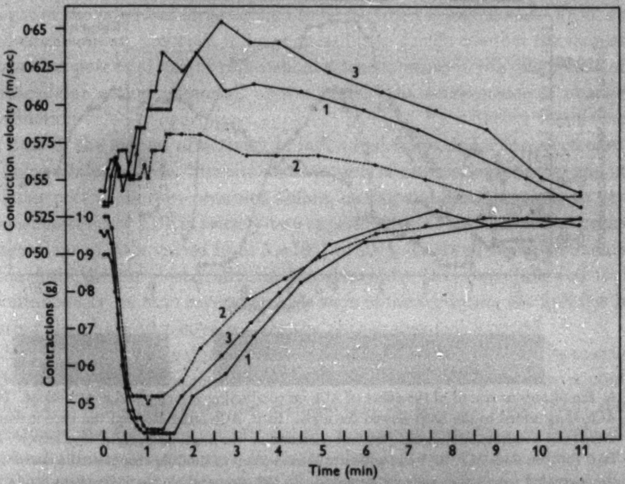

Fig. 3. Simultaneous records of effect of ACh on conduction velocity (upper records) and contractions (lower records). Solid lines, spontaneously beating auricles; broken lines, driven auricle. The experiments were carried out in the order 1, 2, 3.

(3) Some increase in conduction velocity still occurred in the presence of ACh when the atria were driven at a constant rate. The increase was, however, always less than that observed in the spontaneously beating auricle (Fig. 3). In contrast to the effects of frequency on conduction velocity, the reduction in the size of contractions by ACh was not very different in the driven and spontaneously beating preparations (Fig. 3). This would seem to differ from the conclusions of Vane (1957).

If sufficient ACh was given to stop the pace-maker, the conduction velocity during the first beat of recovery was always faster (Fig. 4*B*). If, instead of adding to the bath a single amount of ACh which was rapidly washed away, the drug was added to the reservoir of solution so that the preparation was bathed in a more stable concentration, the conduction velocity increased as before, and remained high until the solution was changed (Fig. 4 *C*). The increase in conduction velocity was not, therefore, merely a phenomenon associated with the phase of recovery.

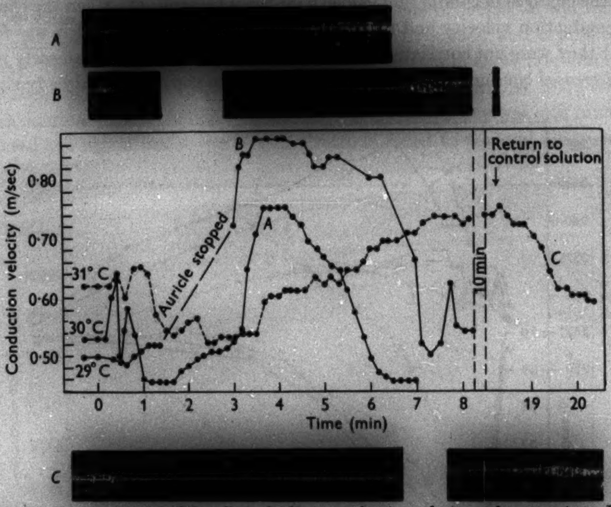

Fig. 4. Simultaneous record of the effect of ACh on conduction velocity and contractions. In *A*, ACh 15 μg added to the bath slowed the atria. In *B*, ACh 80 μg stopped the pace-maker for more than a minute, after which the first contraction was large, and conduction velocity fast. In *C* (broken line) ACh 10^{-8} was added to the reservoir of fluid flowing past the auricle, and the increased conduction velocity persisted. In this preparation the increase in conduction velocity was in each case interrupted by a temporary phase of decreased velocity. The graphs have been plotted on the same time scale as the drum speed.

Phase of decreased conduction velocity

In most preparations conduction velocity began to increase immediately after the addition of ACh, and remained fast until the ACh was washed out, as in Fig. 3. In eight atria, however, conduction velocity began to increase, but the increase was then interrupted by a brief phase of decreased velocity, as shown in Fig. 4. This phase always came to an end soon after the peak effect of ACh was established, and gave place to an increase in velocity which thereafter followed the usual time course. Conditions which would either favour or abolish this phase of decreased velocity did not come to light, but a suggested explanation for the phenomenon is given below.

As a result of the above experiments it was concluded that the chief effect of ACh upon conduction velocity in isolated rabbit atria was to produce a prolonged increase in velocity, though in some preparations this might be interrupted by a short-lasting phase of decreased velocity. A faster conduction velocity might be the consequence of a swifter rate of rise of the upstroke of the intracellular action potential to the same height, or of an increase in the absolute height of the potential in the same time. In the presence of ACh an increase of more than 10% in the height of the externally recorded action potential was observed in nineteen atria. In three preparations, in the presence of a sufficient concentration of ACh to stop the pace-maker, the height of the action potential was decreased. In the remainder there were no changes in the height of the potential large enough to be regarded as significant. From external records alone, however, it was impossible to decide whether the increase in the action potential height represented a true increase in the magnitude of the intracellular action potential, or was merely the consequence of changes in conduction velocity causing an apparent increase, by reducing the dispersion in time of the arrival of activity in individual fibres under the electrode.

Intracellular records. Simultaneous records of conduction velocity and intracellular potentials were obtained before, during and after exposure to various concentrations of ACh in twenty-two experiments in seven atria. The peak concentrations of ACh varied from 3×10^{-7} to 10^{-4}. In six of the experiments the atria were driven electrically; in the remainder they were allowed to beat spontaneously, so that several records were obtained during the first few beats during the phase of recovery after the pace-maker had stopped.

Measurements made during the period when ACh was exerting its maximum effect (as judged by the reduction in pace-maker frequency and size of contraction, i.e. 1·5–3·5 min after the introduction of ACh into the bath) showed invariably the following effects: (*a*) an increase in the absolute height of the intracellular action potential; (*b*) an increase in the rate of rise of the upstroke of the intracellular action potential; (*c*) an increase in conduction velocity. With one exception, in which the concentration of ACh was 10^{-6}, there was also (*d*) an increase in resting potential. Photographs of intracellular potentials on a fast sweep, together with simultaneous records of conduction velocity, taken from two experiments before and during the action of ACh, are shown in Fig. 5.

The upstroke of the intracellular action potential is S-shaped, and can be divided into three portions. A slow foot (*AB* in Fig. 6I), followed by a fast linear section *BC*, and ending with a section slowly rising to the summit *CD*. The maximum rate of rise was obtained from the tangent to *BC*, and in the case illustrated was 60 V/sec. The mean rate of rise could be taken as the vertical distance *AD*, divided by the horizontal distance *AD*, and was 30 V/sec. It was found experimentally that these two statistics varied independently. For example, in the initial phase immediately after exposure to ACh the peak voltage was reached earlier, mainly as a result of a shortening of the third terminal section *CD*, without any change necessarily taking place in the maximum rate of rise, in *BC*. In Fig. 6 II, as an illustration, the mean rate of rise was 35 V/sec, while the maximum rate of rise has been decreased to 48 V/sec. The distinction was not without importance because there is evidence, in Purkinje tissue at least (Weidmann, 1955), that the maximum rate of rise reflects the availability of 'sodium carrier'. An increase in conduction

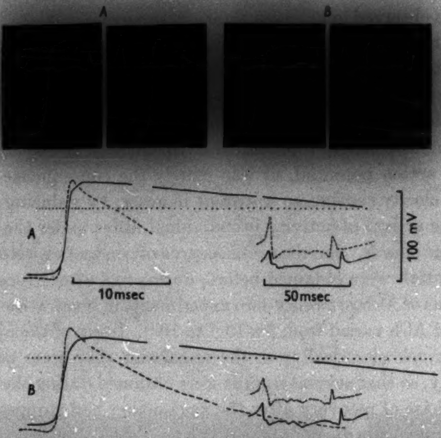

Fig. 5. Simultaneous records of conduction velocity and intracellular potentials in spontaneously beating atria. ——, normal beat; – – – –, at the peak of the effect of ACh 50 μg added to the bath. The tracings have been made from the photographic records shown above.

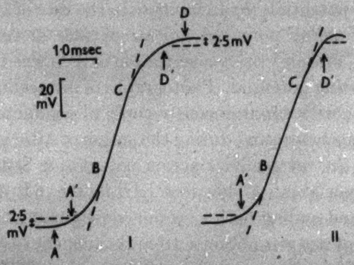

Fig. 6. Diagram of parameters measured. The maximum rate of rise of the upstroke of the intracellular action potential was calculated from the tangent to *BC*. The mean rate of rise was taken as $\frac{(AD-5)}{A'D'}$ mV/msec, where *A'* and *D'* were points at which the potential crossed a line 2·5 mV above and below its most negative and positive values respectively. These two statistics varied independently. In II the maximum rate of rise was slower than in I, but the mean rate of rise was faster.

velocity could be produced by an increase in the *mean* rate of rise of the intracellular action potential, without necessarily any change occurring in the slope of the steep middle portion of the spike, and it was of interest to know whether there was in fact any increase in the maximum rate of rise.

In practice the exact beginning and end of each upstroke was difficult to estimate. To calculate the mean rate of rise, therefore, the horizontal distance in milliseconds was measured between the points A' and D' (Fig. 6I) at which the rising curve cut a horizontal line 2·5 mV above the most negative value and 2·5 mV below the most positive value of the potential respectively. The mean rate of rise was thus calculated as $\frac{(AD-5)}{A'D'}$ mV/msec.

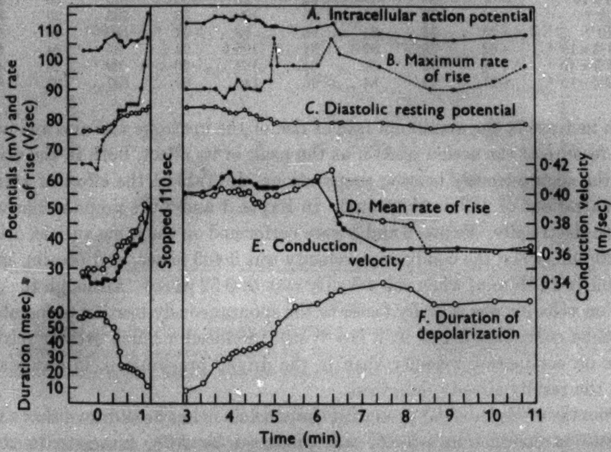

Fig. 7. Parameters of intracellular potential from a single fibre before, during and after the action of ACh. Temp. 31° C. Ordinate: top left, the same numerals apply to millivolts (curves *A* and *C*) and V/sec (curves *B* and *D*); bottom left, msec (curve *F*, duration of action potential at half the height); right, m/sec (curve *E*, conduction velocity). Conduction velocity is correlated with the mean rate of rise.

In Fig. 7 are presented the results of an experiment in which a microelectrode stayed in a single fibre for more than an hour without a fall in the resting potential. The graph records resting and action potentials, the mean and maximum rates of rise of the upstroke of the action potential, the width of the action potential in milliseconds at half its height, and conduction velocity. It is clear that the duration of the potential bore no relation to conduction velocity. This was a general finding in all the experiments, and is evidence against the suggestion of Ramos & Rosenblueth (1947) that the duration of the action potential influences conduction velocity. The correlation between conduction velocity and the mean rate of rise is surprisingly close in view of the fact that the potential is recorded from a single fibre, while the conduction velocity represents the average activity of thousands of fibres.

TABLE 1. The effects of ACh on conduction velocity, with simultaneous records of changes in the intracellular resting and action potentials, and in the rate of rise of the upstroke of the latter

	Change in conduction velocity	Resting potential		Action potential		Rate of rise			
						Max.		Mean	
ACh concn.	(%)	Before (mV)	At peak (mV)	Before (mV)	At peak (mV)	Before (V/sec)	At peak (V/sec)	Before (V/sec)	At peak (V/sec)
1. 3×10^{-7}	+ 6	80	90	91	99	54	85	25	36
2. 10^{-6}	+10	77	81	90	100	53	103	25	38
3 10^{-6}	+27	74	81	94	99	64	110	40	51
4. $1{\cdot}3 \times 10^{-5}$	+62	75	83·5	94	106·5	64	85	24	54
5. $1{\cdot}3 \times 10^{-5}$	+ 8	76	80	94	104	56	89	26	42
6. $2{\cdot}6 \times 10^{-5}$	+21	79	84	96	115	69	107	28	63

An increase in the maximum rate of rise of the upstroke was also an invariable feature of the action of ACh at the peak of its effect, both in the driven and the spontaneously beating preparations. In Table 1 the effect of various concentrations of ACh is illustrated. In Expts. 1 and 2 the preparations were driven electrically. Expts. 2 and 3 were performed on the same auricle, driven at 120/min, when the conduction velocity was 0·403 m/sec, and beating spontaneously at 85/min, when the velocity rose to 0·52 m/sec. Although the conduction velocity was initially faster in the spontaneously beating preparation, the same concentration of ACh (10^{-6}) still produced a much greater relative effect on conduction velocity than in the driven preparation, in accordance with the results already described.

From the evidence so far presented the conclusion has been drawn that a true increase in conduction velocity was produced by ACh, associated with an augmentation of the absolute height of the action potential, and of the mean and maximum rates of rise of the upstroke of the potential. Attention may now be turned to seeking an explanation for the phase of reduced conduction velocity sometimes seen during the initial period of exposure to ACh.

Phase of decreased conduction velocity

This was not due to an early reduction in the rate of rise of the upstroke of the active potential preceding the increase described above. A phase of decreased velocity was observed in six of the twenty-two experiments in which simultaneous intracellular potential records were obtained. In every case the rate of rise of the upstroke was increased during the phase of decreased velocity. In ten of the twenty-two experiments there was an initial fall in the height of the action potential, of 3, 3, 3, 3, 4, 5, 8, 8, 8 and 14 mV. In every experiment the height subsequently increased. The temporary reductions cannot have been the sole factor responsible for the phase of reduced velocity, however, because in six of them (including two when the height was reduced by 8 mV) there was no phase of reduced velocity. In these experiments presumably the concomitant increase in the rate of rise of the upstroke more than counterbalanced the fall in the absolute height. Further, in two experiments, the phase of slower conduction velocity still occurred when the height of the action potential was not reduced. One of these two experiments is illustrated in Fig. 8. The increase in resting potential preceded the increase in action potential, and the conduction velocity was reduced. When the action potential itself increased, then the trend of the conduction velocity reversed, and it, too, was increased.

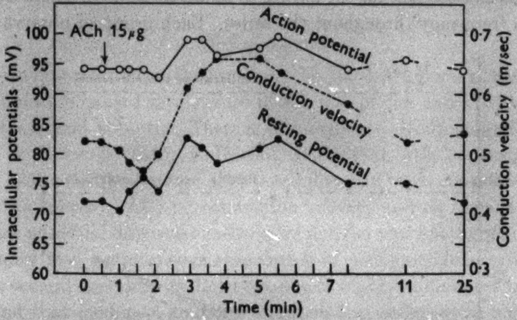

Fig. 8. Record of intracellular potentials during a phase of reduced conduction velocity. The increase in resting potential preceded the increase in the action potential, and the interval corresponded approximately with the phase of reduced conduction velocity.

Detailed examination of all the simultaneous measurements of potential, rates of rise, and conduction velocity has led to the following conclusions. (*a*) The first observable changes in the shape of the intracellularly recorded potential after exposure to ACh were a shortening of its duration, which had no influence on conduction velocity, and a shortening of the third upper segment of the upstroke of the action potential, leading to an earlier peak and an increase in the mean rate of rise of the upstroke—this tended to increase conduction velocity. (*b*) The next changes were an increase in resting potential and an increase in the maximum rate of rise of the upstroke, perhaps implying an increased availability of sodium carrier. (*c*) The absolute height of the action potential was increased, but this increase was sometimes preceded by a temporary decrease. If the decrease was large, or if the increase in resting potential occurred before that of the action potential, there was sometimes a temporary fall in conduction velocity, in spite of the fact that the rate of rise of the upstroke of the action potential was always faster.

The effects of changes in temperature

The above conclusions were open to the criticism that at 30–31° C the resting potential might have fallen so low that the preparation was 'unphysiological', and that at a higher temperature the resting potential might be closer to the equilibrium potential for K, and so be unable to increase. Fig. 9 presents the results of five sets of observations, carried out in the order 1–5, at different temperatures. In order to avoid the complicating factor of the changes in the frequency of the pace-maker which would have occurred if the preparation had been permitted to beat spontaneously, the atria were driven throughout at 175 beats/min, a rate which proved faster than the natural pace-maker at 35·5° C, yet not too fast for the auricle to follow at 26·5° C. There was thus no change in frequency throughout the series. Each group of observations con-

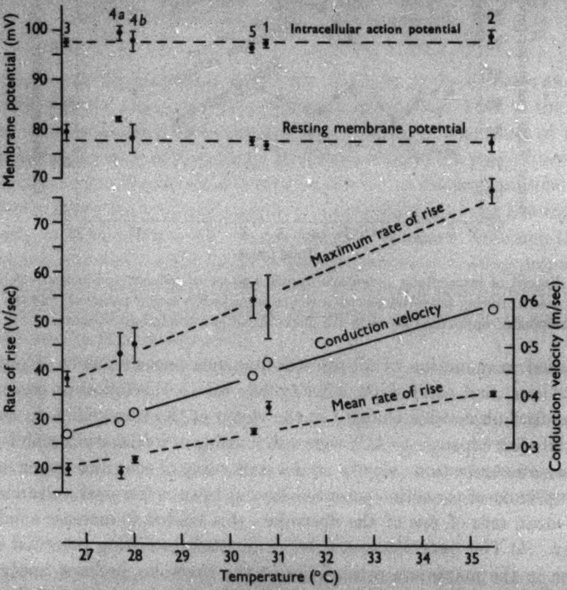

Fig. 9. The effect of temperature upon conduction velocity and intracellular potential. Five sets, of six observations each, were carried out in the order 1–5. Group 4 has been divided into two groups of three because the temperature drifted 0·3° C during the observations. There was no significant change in resting or intracellular action potentials, but the conduction velocity and the mean and maximum rates of rise of the action potential had a Q_{10} of 2. S.E. indicated for each group of observations. The measurements of conduction velocity were almost identical within each group.

sisted of six separate entries. When the temperature was changed the preparation was left half an hour at the new temperature before the observations were made. (Group 4 was divided into two, because the temperature had crept up 0·3° C during the observations.) It is evident that over the range 26·5–35·5° C there was no significant change in the resting or action potentials. The conduction velocity, on the other hand, and the mean and maximum rates of rise of the upstroke of the action potential, had a Q_{10} of about 2. In another preparation, in which simultaneous measurements of conduction velocity and intracellular potentials were made during exposure to ACh at 35° C, the usual increases occurred in the resting and action potentials, in the conduction velocity, and in the mean and maximum rates of rise of the upstroke.

The effect of adrenaline

It has been reported that in the frog sinus (Hutter & Trautwein, 1956) sympathetic stimulation caused an increase in the rate of rise and in the absolute height of the action potential. There is also evidence (Hoffmann, Hoffmann, Middleton & Talesnik, 1945; McDowall, 1946) that ACh can excite post-ganglionic sympathetic neurones. The possibility had to be considered, therefore, that the effects of ACh on conduction velocity and on the shape of the intracellular potential described above were not the true effects of ACh itself, but secondary to a simultaneous stimulation of post-ganglionic sympathetic nerves. In several experiments, however, one of which is illustrated in Fig. 10, it was found that moderate amounts of adrenaline, sufficient to increase the force of contraction by 50–100%, had no effect on conduction velocity, nor on the mean and maximum rates of rise of the intracellular action potential. Higher concentrations decreased the conduction velocity and the rate of rise of the action potential. The results of two other experiments are given in Table 2. In both these experiments the atria were driven at 175 beats/min, a rate which proved fast enough to prevent the natural pace-maker from taking over even in the presence of adrenaline. In both experiments there was an increase in the absolute height of the action potential.

It was shown recently by Bertler, Carlsson & Rosengren (1956) that the injection of reserpine 5 mg/kg intravenously into rabbits was followed within 16 hr by a virtually complete removal of catechol amines from their hearts. This observation made it possible to test in another way whether the excitation of post-ganglionic fibres could be involved in the action of ACh, since a preparation could be obtained from which all sympathetic transmitter had been removed. Accordingly, a rabbit was injected intraperitoneally with 0·5 mg/kg of reserpine, and after 24 hr intravenously with 5 mg/kg of reserpine. This procedure has been shown to reduce the catechol amine content of auricles to less than 0·3% of their original value. Sixteen hours after the second injection of reserpine the atria were set up, and simultaneous measurements of conduction velocity and intracellular potential were made during exposure to various concentrations of ACh. The results did not differ (Table 2) from those obtained in the other experiments. It has been concluded, therefore, that the changes in conduction velocity and intracellular potential reported above were the consequence of the effects of ACh, and not secondary to a simultaneous stimulation of post-ganglionic sympathetic fibres.

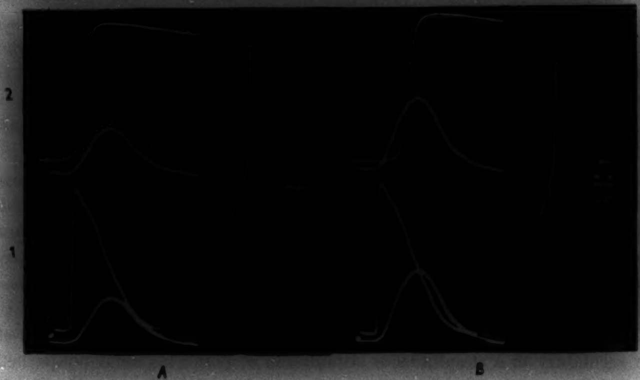

Fig. 10. The effect of adrenaline. Photographs of intracellular potential and contraction during successive beats (1 and 2). The lower trace in each frame is the transducer record of contraction. Immediately above is the intracellular potential record. The latter has been photographed at slow and fast sweep speeds (frames 1 and 2). On the right of frame 2 the vertical trace shows action potentials recorded from two external electrodes, and on the extreme right the time at which each photograph was taken is recorded to within 0·1 sec. The preparation followed electrical stimuli throughout at 175/min. *A*, normal beats (at 20 min 40·7 sec); action potential height, 94 mV. *B*, in the presence of adrenaline 0·3 μg/ml. (at 21 min 38·7 sec); the action potential was now 97 mV and the contraction had increased by 50 %; the conduction velocity was unchanged. The apparent increase in the rate of rise of the upstroke is an illusion due to the increased height.

TABLE 2. Comparison of the effect of adrenaline with the effect of ACh on a preparation from which sympathetic transmitter had been removed by previous injections of reserpine

Drug	Change in conduction velocity (%)	Resting potential (mV)		Action potential (mV)		Mean rate of rise (V/sec)		Max. rate of rise (V/sec)		Change in contraction (%)
		Before	At peak	Before	At peak	Before	At peak	Before	At peak	
Adr 3×10^{-7}	0	77·5	77·5	96	103	35	35	68	68	+ 60
Adr $1{\cdot}3 \times 10^{-6}$	0	79	79	90	94	29·8	28	61·5	42	+240
	Atria from rabbit previously injected with reserpine									
ACh 10^{-6}	+10	78	81	88	99	28	32	64	100	−40
ACh 10^{-4}	+57	76	83	87	97	24	54	56	110	−79

DISCUSSION

Previous evidence concerning the effect of vagal stimulation or of ACh on conduction velocity in mammalian atrial muscle has led to conflicting conclusions. Lewis *et al.* (1920) found that vagal stimulation caused no change in conduction velocity in dog atria, either when beating spontaneously or when driven electrically at a constant frequency. Brooks *et al.* (1956), on the other hand, also working with dogs, observed that conduction velocity was increased by vagal stimulation, but Burgen & Terroux (1953*b*) found that ACh and carbamylcholine reduced conduction velocity in isolated strips of atria taken from cats. Webb & Hollander (1956) found that the action potential in rat atria was about 70 mV and was slightly increased by ACh. They observed that ACh reduced conduction velocity but did not cause any fall in the height of the intracellular action potential. In the present series of experiments with isolated rabbit atria, it was found that the main effect of ACh was to increase conduction velocity, though in some auricles the increase was interrupted by a temporary phase of decreased velocity when the concentration of ACh was high. Simultaneous records of conduction velocity and intracellular potential have provided an explanation for these effects.

ACh caused an increase in the resting potential and in the rate of repolarization in the rabbit atrium, as has already been reported in the cat and dog. At the peak of the effect of ACh there was an increase both in the absolute height and in the rate of rise of the intracellular action potential. These observations provided an adequate explanation for the increased conduction velocity. The increase in the rate of rise of the upstroke of the action potential was due not merely to a shortening of the slower terminal portion of the spike, but there was also a steepening of the fast central part, implying an increased rate of entry of sodium.

The increases in conduction velocity developed gradually and followed a characteristic course. Sudden changes in the interval between the recorded action potentials did also occur, but were easily recognized as shifts in the pathway of conduction or occasionally in the site of the pace-maker. The externally recorded action potentials frequently increased in size in the presence of ACh, and in view of the results of the intracellular recordings it is likely that these changes represented true increases in height of the intracellular action potential, and not just a reduction in the dispersal of individual potentials caused by conduction velocity changes.

When the atria were stimulated electrically it was found that there was an inverse linear relation between conduction velocity and the frequency of stimulation. The increase in conduction velocity in the presence of ACh was not entirely due to the concomitant slowing of the pace-maker, however, because increases still occurred when the atria were driven at a constant frequency, though the increases were less than in spontaneously beating preparations.

The finding of an inverse relation between frequency and conduction appears to differ to some extent from previous evidence. Prinzmetal, Corday, Brill, Oblath & Kruger (1952) did observe such a relation in dog atria at stimulation frequencies above 350/min, but at lower frequencies the stimulus–potential interval was unchanged. The stimulus–potential interval, however, cannot always be assumed to measure conduction velocity, because changes in the latter might be concealed by opposite changes in latency. Brooks *et al.* (1956) stated that in dog atria there was no change in conduction velocity until the stimulation frequency reached 250/min. The evidence they presented (Fig. 4*C*, p. 196) did show, nevertheless, in two of the three curves illustrated, a slight slope, correlating frequency inversely with conduction velocity, at frequencies between 100 and 240/min. It is possible that the upstroke of the action potential involves a reaction requiring a reagent drawn from a source common to the mechanism of contraction. When contractions are more frequent a reduction in the source might be revealed as a slowing of the upstroke. Such a phenomenon would be likely to occur at lower frequencies in an isolated preparation than in the better oxygenated whole atrium supplied with blood.

In some auricles high concentrations of ACh produced a temporary fall in conduction velocity before the usual increase. Simultaneous recordings of conduction velocity and intracellular potential showed that this phase of reduced velocity was not due to any slowing of the rate of rise of the upstroke of the action potential, which was invariably faster in the presence of ACh. Hutter & Trautwein (1956) explained the decrease of conduction velocity which they observed after vagal stimulation in the frog sinus as due to a fall in the height of the action potential. So far as the phase of reduced conduction velocity in mammalian atria is concerned, a fall in the height of the action potential cannot account for all the facts. Burgen & Terroux (1953*b*) observed that carbamylcholine always reduced conduction velocity in cat auricle strips, but they stated 'the magnitude of the active membrane potential was unchanged except with the highest concentrations used (50–100 μg/ml.), where some of the action potentials were reduced.' In the present experiments a temporary reduction in the height of the action potential was observed on six occasions when the conduction velocity was not reduced and even increased, and on two occasions conduction velocity was reduced when the height of the action potential was not. In these latter cases it was noted that the increase in resting potential slightly preceded the increase in height of the action potential. It has been concluded that conduction velocity is influenced not only by the rate of rise and the height of the action potential, but also by the time at which the increases in these parameters occur relatively to the increase in resting potential.

The absolute height of the action potential was always increased by ACh at the peak of its effect. Burgen & Terroux (1953*b*) never observed an increased 'overshoot' in cat atria, and they stated that 'the rate of rise is little affected', though some increase in the rate of rise is indicated in their Table 2. Hoffman & Suckling (1953) also found an increased rate of rise of the action potential in dog atria after vagal stimulation. The rates of rise in rabbit atria were approximately five times as fast as those reported for the cat by Burgen & Terroux (1953*a*).

Hutter & Trautwein (1956) (in experiments in which the pace-maker frequency was free to increase) observed that both the rate of rise and the height of the action potential were augmented by *sympathetic* stimulation in the frog sinus, and the possibility had to be considered that the results described here were due not to ACh itself, but to stimulation by ACh of post-ganglionic sympathetic neurones. In the experiments described above the frequency of contractions was kept constant, and in these circumstances it was found that adrenaline, although increasing the height of the action potential slightly, did not change the conduction velocity or the rate of rise of the upstroke, and higher concentrations actually reduced them. Further, the usual increases in conduction velocity and rate of rise after exposure to ACh still occurred in atria from which all sympathetic transmitters had been removed by previous treatment with reserpine. It was concluded, therefore, that simultaneous excitation of post-ganglionic sympathetic neurones was not involved.

Previous studies of the effects of temperature changes on various cardiac tissues (Woodbury, Hecht & Christopherson (1951) in frog ventricle, Trautwein (1953) and Coraboeuf & Weidmann (1954) in mammalian Purkinje fibres, Burgen & Terroux (1953*a*) in cat auricle) concur in concluding that there is little alteration in the resting or intracellular action potential between 25° and 37° C, and the present findings have shown that this is also true of the rabbit atrium. So far as the rate of rise of the upstroke of the action potential is concerned, however, there would appear to be some disagreement with the series of observations of the effects of temperature carried out on mammalian auricle by Burgen & Terroux (1953*a*) who stated 'with fall of temperature the rate of rise of the action potential was affected to only a trivial extent between 38° and 24° C'. In the experiments described here it was observed that between 26·5° and 35·5° C there was no significant change in either the resting or action potentials, but the conduction velocity and the rate of rise of the upstroke both had a Q_{10} of about 2·0. The mechanism responsible for ionic transport during diastole evidently has a big margin of safety, being able to maintain ionic concentration differences at their limiting value over a wide range of temperature. The process preceding or accompanying the flow of ionic current during activity, on the other hand, at any rate in rabbit atria, involved a chemical reaction whose rate was highly temperature-dependent. This would be consistent with the inverse relation between conduction velocity and frequency of contraction, if the reactions involved in ion transport during membrane activity and in contraction shared a common reagent. Both contraction and conduction velocity are very sensitive to a reduction in the supply of oxygen. The observation that ACh increases the maximum rate of rise of the action potential is thus of interest, as it implies an influence on the availability of sodium carrier, either directly or as a consequence of the increase in resting potential.

A series of papers in the last few years (Burgen & Terroux, 1953*b*; Fatt, 1954; del Castillo & Katz, 1955; Hutter & Trautwein, 1956; Trautwein, Kuffler & Edwards, 1956) has led to the view that the resting potential in atrial muscle is lower than that theoretically calculable from the ratio of the internal and external concentrations of K, and it has been suggested that ACh opens channels in the membrane wide enough to permit a passive efflux of K, while remaining sufficiently narrow to impede the passive influx of Na. The intracellular potential thus becomes more negative, because the less restricted diffusion of K permits the resting potential to approach the value it could theoretically attain if K were at equilibrium. A similar argument may now be advanced in respect of Na. For since, in the presence of ACh, the intracellular action potential may increase from 97 mV (-79 to $+18$) to as much as 115 mV (-84 to $+31$), to take an extreme example, the implication is that during normal activity the 'overshoot' is well below the equilibrium potential for Na.

The considerable literature concerning the effects of vagal stimulation upon auricular arrhythmias has been reviewed by Prinzmetal *et al.* (1952). It was recently shown by Burn, Vaughan Williams & Walker (1955) that in the dog heart–lung preparation atrial fibrillation could be turned on and off at will by a combination of electrical stimulation with the infusion of ACh. It is possible that the increase in the rate of rise of the action potential produced by ACh, representing a greater availability of sodium carrier, is concerned in the phenomenon. If so, the ability of potassium to arrest fibrillation would be readily explained, since the accompanying fall of membrane potential decreased the rate of rise of the upstroke (Weidmann, 1955, 1956), although the phase of repolarization was much more rapid. Burn, Gunning & Walker (1957) found that adrenaline and noradrenaline could antagonize the development of fibrillation. Webb & Hollander (1956) reported that adrenaline prolonged the duration of the intracellularly recorded action potential in rat atria. Although they did not measure the rate of rise of the potential, they observed that conduction velocity was reduced by adrenaline, as found here. It is possible that a reduced availability of sodium carrier is concerned in the antagonism of adrenaline to the production of fibrillation in atrial muscle.

SUMMARY

1. ACh increased conduction velocity in isolated rabbit atria, although in a few preparations the increase was interrupted by a temporary phase of decreased velocity.

2. There was an increase in the rate of rise and absolute height of the intracellular action potential in the presence of ACh, which could account for the increased conduction velocity. There was also an increase in resting potential and rate of repolarization.

3. In some experiments a temporary fall in the height of the intracellular action potential preceded the usual increase. This could not wholly account for the phase of decreased conduction velocity, which sometimes occurred in the absence of a fall in the height of the intracellular action potential. In these cases it was observed that the increase in resting potential preceded that of the intracellular action potential.

4. Evidence was presented that the above effects were truly those of ACh, and not due to stimulation by ACh of post-ganglionic sympathetic neurones.

5. There was an inverse linear relation between conduction velocity and the frequency of contraction.

6. Between 35·5° and 26·6° C there was no significant change in the resting or intracellular action potentials. Conduction velocity, however, and the rate of rise of the intracellular action potential had a Q_{10} of about 2.

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BLOOD VOLUMES OF ESKIMOS AND WHITE MEN BEFORE AND DURING ACUTE COLD STRESS

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Several investigators have shown that average blood and plasma volumes of Eskimos are greater than are those found in white subjects living in a temperate climate. This evidence has recently been reviewed by Bass & Henschel (1956). Other reports have shown that in Eskimos the blood flow through the hand and forearm was greater than in a control group of medical students at rest in a room at 20° C (Brown & Page, 1952; Brown, Hatcher & Page, 1953) and it has also been noted that during a test exposure to cold a greater reduction of blood flow through the hand and forearm occurred in the Eskimos than in the white controls.

This report will present the results of more recent studies that have been made of the blood volume changes which occur in the two groups during a test cold exposure.

METHODS

The Eskimo subjects were six adult males found free of disease on a thorough medical examination and known from inquiry to be making their living by hunting and trapping in the traditional Eskimo fashion. The observations on the Eskimos were made at Coral Harbour, Southampton Island, N.W.T., during July and August 1954 when the mean daily maximum temperature was about 12° C and the mean daily minimum about 3·5° C. The white men were medical students and internes at Queen's University, and the observations on them were made in September and October 1954 at Kingston, Ontario. The temperature of the rooms used for the experiments was $20 \pm 1^\circ$ C and the relative humidity 47–57%. Air movement was kept constant by an electric fan 8 ft. from the feet of the subject. Each subject was dressed in the same flannel drawers, trousers, shirt and socks and lay supine in bed with head and thorax raised to an angle of 45°. The age, height and weight of each subject are given in Table 1.

Two series of determinations of plasma volume were made on the Eskimos using the methods of Allen & Semple (1951) and Allen (1953). In the first (preliminary) series all six subjects were used and six blood samples obtained from each subject at 15 min intervals after the injection of T-1824. These observations showed that for 90 min after injection the relation between time and plasma concentration of dye was linear (Fig. 1A). The second series consisted of seven experiments on five of the subjects used in the first series and blood samples were secured every 15 min for

1 hr after dye injection, to redetermine control volumes. Then the right arm was immersed to a level just above the elbow in a water-bath at 5±0·5° C. The exposure to cold was continued for 1 hr and during this period blood samples were secured at 20 min intervals. Two more blood samples were secured at 20 min intervals during the recovery period. Dye concentrations were determined in the control samples and in the samples secured 20 min after the start of the immersion. Any change in plasma volume during the 20 min of cold exposure was determined from the concentration of dye. If no change in plasma volume occurred, the dye concentration in the plasma could be expected to decrease at the same rate as in the control period, and it was assumed that deviation from the expected disappearance rate indicated a change in plasma volume (Fig. 1 B).

TABLE 1. Measured and predicted plasma volumes of Eskimo and white subjects

					Plasma volumes			
					Measured, M		Predicted*, P	
Subject	Expt. no.	Age (yr)	Height (m)	Weight (kg)	(l.)	(ml./kg)	(l.)	M/P (%)
Eskimos								
Okalik	1	34	1·64	68·0	3·46	50·9	2·80	123·6
	2			68·3	3·45	50·5	2·81	122·8
	3			68·0	3·65	53·7	2·80	130·4
Evalayuk	4	31	1·66	61·7	2·95	47·8	2·67	110·5
	5			59·7	2·94	49·2	2·63	111·8
	6			60·0	3·03	50·5	2·63	115·2
Akat	7	23	1·61	64·9	2·85	43·9	2·66	107·1
	8			63·5	3·00	47·2	2·63	114·1
Pootolik	9	20	1·63	61·8	3·54	57·3	2·62	135·1
	10			62·0	3·53	56·9	2·63	134·2
Tatti	11	28	1·87	77·4	4·62	59·7	3·55	130·1
	12			75·3	4·60	61·1	3·50	131·4
Napyuk	13	29	1·66	65·4	3·22	49·2	2·77	116·2
				Means	3·45	52·1	2·82	121·7
Whites								
A. S.	I	34	1·74	68·0	2·86	42·1	3·01	95·0
D. M.	II	35	1·79	76·6	3·26	42·6	3·34	97·6
H. H.	III	35	1·74	66·3	3·07	46·3	2·96	103·7
S. B.	IV	31	1·86	73·7	3·39	46·0	3·43	98·8
M. M.	V	22	1·83	63·5	2·92	46·0	3·09	94·5
C. W.	VI	22	1·85	79·8	3·92	49·1	3·56	110·1
				Means	3·24	45·4	3·23	100·0

* Predicted plasma volumes were calculated by multiplying the predicted blood volumes (see Table 2) by 0·575, which is the reciprocal of the average human male haematocrit reading.

Wintrobe tubes were used to measure the haematocrit values of all blood samples, and the cell percentages so obtained were multiplied by the factor 0·96 to correct for trapped plasma and by the factor 0·91 to correct for unequal cell distribution in the blood stream (Chaplin, Mollison & Vetter, 1953). Haematocrits were also corrected for cells removed in previous blood samples (Allen & Semple, 1951). Each plasma sample was frozen as soon as the dye concentration had been determined, and on return to Kingston the protein concentrations were determined by measuring the total nitrogen and the non-protein nitrogen by micro-Kjeldahl methods.

A single control determination of plasma volume was made on each white subject during the 40 min that preceded immersion of the arm, and during this time blood samples were secured at 10 min intervals. In all other respects experiments on the white subjects were identical to those described for the Eskimos.

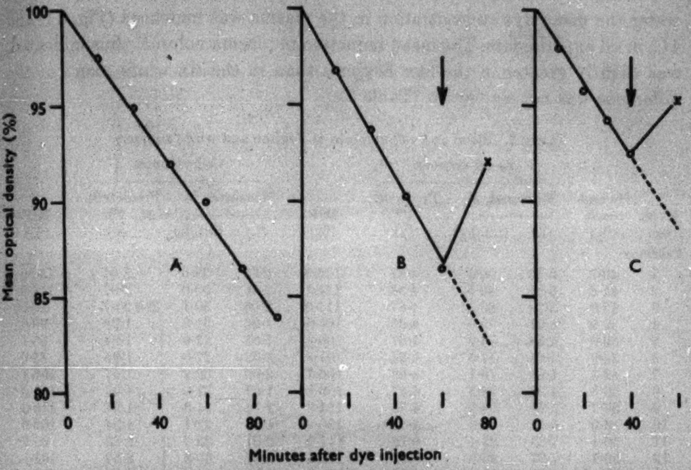

Fig. 1. Mean optical densities (expressed as percentages of optical densities at zero time) of serial plasma samples after injection of T-1824 at zero time. Each percentage plotted represents a mean value (at given time) for all experiments. A, six experiments on six Eskimo subjects, no cold immersion; B, seven experiments on five of the subjects in A, ↓ indicates time of arm immersion in 5° C water-bath; C, six experiments on six white men, arm immersion as in B.

RESULTS

Control measurements. Table 1 shows that Eskimo plasma volumes were significantly greater ($P < 0{\cdot}01$) than those of the white men when expressed in terms of body weight. In terms of surface area the plasma volumes of the six Eskimos ranged from 1·75 to 2·28 l./m^2 (mean 1·98) as compared with the range in white men of 1·58–1·92 l./m^2 (mean 1·70) ($P < 0{\cdot}05$). The Eskimo haematocrit values (Fig. 2 and Table 2) were lower than those of the white men and the difference was roughly proportional to the difference between the plasma volumes. There was, therefore, no significant difference between the Eskimos and white men with respect to total red cell volume, but the greater plasma volume of the Eskimos resulted in this group having a larger total blood volume.

Protein concentrations (Fig. 2) of Eskimo plasma under control conditions ranged from 6·97 to 8·48 g/100 ml. (mean 7·45) which were significantly higher ($P < 0{\cdot}02$) than the concentrations in the plasma samples obtained from the white men, which ranged from 5·98 to 7·21 g/100 ml. plasma (mean 6·62).

Post-immersion measurements. After 20 min of immersion of the arm in cold water the mean dye concentration in the plasma was increased (Fig. 1B and 1C) in all experiments. The mean reduction in plasma volume, thus indicated, was slightly greater in the five Eskimos than in the six white men but the difference was not significant (Table 3).

TABLE 2. Blood and cell volumes of Eskimo and white subjects

		Blood volumes				Cell volumes			
	Haematocrit	Measured, M		Predicted, P*	M/P	Measured, M		Predicted, P†	M/P
Expt. no.	(%)	(l.)	(ml./kg)	(l.)	(%)	(l.)	(ml./kg)	(l.)	(%)
Eskimos									
1	43·7	6·15	90·4	4·87	126·3	2·69	39·6	2·07	130·0
2	41·6	5·91	86·5	4·88	121·1	2·46	36·0	2·07	118·8
3	36·0	5·70	83·8	4·87	117·0	2·05	30·1	2·07	99·0
4	39·9	4·91	79·6	4·65	105·6	1·96	31·8	1·98	99·0
5	36·0	4·59	76·9	4·57	100·4	1·65	27·6	1·94	85·1
6	34·9	4·65	77·5	4·58	101·5	1·62	27·0	1·95	83·0
7	42·3	4·94	76·1	4·63	106·7	2·09	32·2	1·97	106·1
8	39·6	4·97	78·3	4·57	108·8	1·97	31·0	1·94	101·5
9	37·7	5·68	91·9	4·56	124·6	2·14	34·6	1·94	110·3
10	36·0	5·52	89·0	4·57	120·8	1·99	32·1	1·94	102·6
11	36·1	7·23	93·4	6·18	117·0	2·61	33·7	2·63	99·2
12	36·7	7·27	96·5	6·09	119·4	2·67	35·3	2·59	103·1
13	39·6	5·33	81·5	4·82	110·6	2·11	32·3	2·05	102·9
Mean	38·5	5·60	84·7	4·91	113·8	2·16	32·6	2·09	103·1
Whites									
I	42·5	4·97	73·1	5·23	95·0	2·11	31·0	2·22	95·0
II	37·2	5·19	67·7	5·81	89·3	1·93	25·2	2·47	78·1
III	42·2	5·31	80·1	5·15	103·1	2·24	33·8	2·19	97·7
IV	43·5	6·00	81·4	5·97	100·5	2·61	35·4	2·54	97·2
V	43·7	5·19	81·7	5·38	96·5	2·27	35·7	2·29	99·1
VI	37·5	6·27	78·6	6·20	101·1	2·35	29·4	2·64	89·0
Mean	41·1	5·49	77·1	5·62	97·6	2·25	31·8	2·39	94·4

* Calculated from the equation: $BV_1 = (0{\cdot}417 \times \text{height}^3_{\text{m}}) + (0{\cdot}045 \times \text{weight}_{\text{kg}}) - 0{\cdot}03$ (Allen *et al.* 1956).

† Calculated from the equation: $CV_1 = (\text{predicted blood volume}) \times 0{\cdot}425$ (average human male haematocrit.)

The haematocrit values of blood samples drawn 20 min after immersion varied considerably, but were on the average slightly higher than in the control period in both Eskimos and white men; but again the difference was not significant. At the end of the exposure to cold the haematocrit values were significantly increased in both groups (Fig. 2). During the recovery period the haematocrit values of the Eskimos immediately began to decrease but did not, on the average, reach control values (Fig. 2). Those of the white men, on the other hand, tended to rise further and remained significantly elevated at the end of the 40 min recovery period. Fig. 2 shows that the test exposure to cold resulted in no significant changes in the protein concentration of plasma of either group, although in the Kingston subjects there was a slight rise during the recovery period.

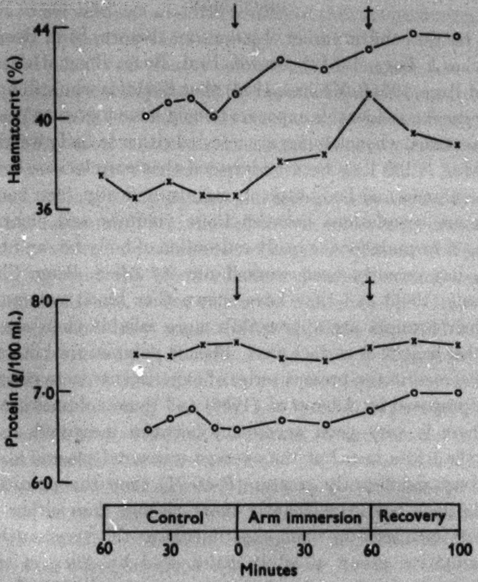

Fig. 2. Changes in mean haematocrit percentages and in mean protein concentrations in plasma during control, immersion and recovery periods. Each point plotted represents the mean (at given time) for seven experiments on Eskimos (×) or for six experiments on white men (○). ↓, start of arm immersion in 5° C water-bath; ‡, end of immersion.

Table 3. Changes in plasma and red cell volumes that resulted from the immersion of one arm in a 5° C water-bath for 20 min. Volumes are expressed as percentages of control volumes

	Eskimos. Changes in			Whites. Changes in	
Expt. no.	Plasma volume (%)	Cell volume (%)	Expt. no.	Plasma volume (%)	Cell volume (%)
2	−6·2	+7·3	I	−5·5	−1·4
3	−0·5	−11·7	II	−13·7	−9·1
5	−12·2	−6·0	III	−5·6	+1·4
6	−17·3	−13·6	IV	−5·9	−0·4
8	−7·7	−2·5	V	−5·5	−5·8
10	−9·8	+8·0	VI	−6·1	+2·3
12	−15·0	−10·5			
Means (7 Expts.)	−9·8	−4·1	Means	−7·1	−2·2
Means (5 Eskimos)	−10·1	−3·4			

DISCUSSION

The present results confirm earlier observations (Brown, Bird, Boag, Delahaye, Green, Hatcher & Page, 1954*a*; Brown, Bird, Boag, Boag, Delahaye, Green, Hatcher and Page, 1954*b*; Meehan, 1954) that Eskimos who still hunt and trap and thereby incur considerable exposure to cold have a greater plasma volume than do Caucasians, when volumes are referred either to body weight or surface area. However, it has long been understood that correlations between blood and plasma volumes and body type or, more specifically, lean body mass, are better than are correlations between these volumes and either weight or surface area. A formula for the ready estimation of body fat, and hence of lean body mass, has recently been worked out by Allen, Peng, Chen, Huang, Chang & Fang (1956) and they have shown that blood volume predictions based on their formula are appreciably more reliable than are predictions based on either weight or surface area. Plasma volumes for both the Eskimos and the white men in the present series of experiments were calculated using the formula proposed by Allen *et al.* (1956) and these volumes are included in Table 1. There is very good agreement between measured and predicted volumes in the white men but the average measured plasma volume of the Eskimo is very significantly greater ($P < 0{\cdot}01$) than the predicted volume. Thus the Eskimos have significantly more plasma than either the control group of white men used in these experiments or the very much larger and more representative group of adult males used by Allen *et al.* (1956) to establish the prediction formula. In an investigation in 1950 by two of the present authors and others (Brown *et al.* 1954*a*, *b*) the average plasma volume in nineteen adult male Eskimos was found to be 3·69 l. and when the Allen formula was applied to the average height and weight of this group the predicted plasma volume was 2·75 l. In the present group the total red cell volumes are not significantly different from the red cell volumes of the white men or from predicted red cell volumes. This result is apparently not in agreement with earlier observations, in which increased red cell volumes were noted (Brown *et al.* 1954*a*, *b*). The haematocrit values in the earlier work were not, however, corrected for either 'trapped' plasma or for unequal cell distribution. When the data are appropriately corrected the average cell volume is appreciably less than first reported, but is still 2·43 l. as compared with a predicted volume of 2·03 l. (+21·2 %). It may be that the Eskimos of the group studied in 1948 were better acclimatized to cold than those of the present group. The Eskimos of Southampton Island are gradually acquiring more of the white man's habits and many of them are leading a more sheltered existence than formerly. To-day many of the group live at the trading post, whereas in 1948 most of them came to the post by water after the break-up of the ice, which occurs early in July, and left for isolated parts of the island well before freeze-up, which can occur as early as mid September. For the present experiments it was difficult to get subjects who had employed themselves during the previous winter entirely at hunting and trapping.

The elevated blood and plasma volumes of the Eskimos are considered by us to be a physiological adjustment to repeated and severe exposure to cold. Bass & Henschel (1956) criticized this conclusion as being largely speculative, and suggested that factors other than acclimatization, such as diet and genetics, may be the cause of the Eskimo high blood volume. There is little evidence that diet *per se* affects blood volume, and indeed there is good evidence that it does not. Thus Lippman (1948) showed that in rats a large increase in protein consumption produced no significant changes in plasma or blood volumes. Davis (1942) reported the blood volumes of eleven normal men living on British war-time rations, a diet relatively rich in carbohydrate and poor in fat and protein, and they were similar to those reported in comparable American studies (Stewart & Rourke, 1941; Noble & Gregersen, 1946), where the subjects lived on a diet much richer in protein and fat. Other large experimental groups existing on very low protein–high carbohydrate diets in Formosa (Allen *et al.* 1956) and Nanking (Gregerson, Lu & Wang, 1949) showed blood volumes that were essentially the same as those from the control group of Kingston students observed in this study. Eskimos in the Canadian Eastern Arctic have a high intake of vitamin A, but the maintenance of a similarly high vitamin A intake for one month in Caucasians has been found by us to have no effect on blood volume (Bird, Brown, Morley & Semple, unpublished). There is also little evidence to suggest that genetics plays a role in Eskimo high blood volume. These people are related to the folk of Asia who, as noted above, have the same relative blood volumes as Caucasians (Gregerson *et al.* 1949; Allen *et al.* 1956). Among the Eskimos themselves those of mixed racial origin showed the same high blood and plasma volumes as the men of pure Eskimo stock. An example in the present series is the Eskimo subject Tatti, 1·87 m (Tables 1 and 2), and other examples have been reported (Brown *et al.* 1954*b*). In our opinion it is the suggestion that either diet or genetics plays a role in this phenomenon which is speculative.

Bass & Henschel (1956) also questioned the assumption that Eskimos are actually cold-acclimatized. There is some cause for such criticism if reports by Stefansson (1921) and Rodhal (1953) are uncritically accepted. The truth is that the summer conditions near Great Bear Lake as described by Stefansson (1921) are quite different from those prevailing in the central and eastern Canadian Arctic. Stefansson (1921) writes '...for six weeks the temperature rose to 90° in the shade nearly every day...the sun does not set and there is no respite through the cooling darkness' (90° = 32·2° C.). The conditions actually encountered at Coral Harbour have been given in the earlier section of this report. Similarly, Rodahl (1953) states that Eskimos spend most of the day in temperatures higher than those found in the white man's house. These reports led Bass & Henschel (1956) to conclude that the Eskimo lives largely in a tropical or subtropical microclimate. In winter the group of Eskimos on whom the present report is based lived outdoors for weeks on end while at work on their trap-lines. For periods in the day they could be described as keeping part of their bodies in a tropical microclimate, but for other periods circumstances do not permit such ideal conditions and there is in fact a good deal of exposure to cold. The temperatures of their living quarters are interesting. Morning temperatures in two igloos in the month of April were between −8·5° and 0° C and in two typical shacks they were between −2·5° and 11° C. Wisely chosen clothing can do much to compensate for a low ambient temperature, but for an Eskimo still living the traditional native life completely to avoid exposure to cold is an impossibility. To be an Eskimo is not necessarily to be acclimatized to cold, but it is our conclusion, for the reasons given, that our selected Eskimo subjects were so acclimatized.

The acute effects of cold stress have been noted by several investigators and are quite different from the acclimatization changes that result from repeated or prolonged exposure. In general a decrease in blood volume has been reported (Conley & Nickerson, 1945; Stein, Elliott & Bader, 1948; Rodbard, Saiki, Malin & Young, 1951; D'Amato & Hegnauer, 1953). Conley & Nickerson (1945) found the decrease was temporary and the volumes tended to return to normal in a few days. When the exposure to cold has been somewhat longer and more severe, there has been evidence that plasma volume possibly increased. LeBlanc, Stewart, Marier & Whillans (1954) exposed men dressed in shorts, socks and shoes to 15·0 ± 0·5° C for 16 days and found the haematocrit values decreased during the exposure. Barlow (1954) made haematocrit readings on soldiers before and after a 23-day winter exercise and reported a 2% decrease. It may also be noted here that Deb & Hart (1956) reported that the blood volume of rats exposed for 5 weeks to 6° C was 22% greater than the blood volume of rats kept at 30° C. The localized acute cold stress used in the present series of experiments resulted, as expected, in decreases in blood volumes of both Eskimos and whites. Table 3 shows that the decrease came about chiefly as a result of decreased plasma volumes, although there was some evidence of lowered red cell volume in most individuals. The adjustments made by the Eskimos were greater and occurred more quickly than those made by the white group, although the differences are not statistically significant. The changes in haematocrit values, which were observed throughout the entire period of exposure to cold, suggested continuing decreases in Eskimo plasma volume; decreases that were more rapid and of greater magnitude than the changes in the plasma volumes of the Kingston group. During the recovery period the return to control levels was also more rapid in the Eskimo group.

Indeed, Fig. 2 shows that haematocrit levels suggested no trend back to control plasma volumes in the Kingston subjects during the 40 min period of observation that followed immersion.

It is interesting that there occurred no significant changes in plasma protein concentrations throughout these experiments (Fig. 2). Rodbard *et al.* (1951) and D'Amato & Hegnauer (1953) have also reported that hypothermia resulted in no change in the total protein concentration of the plasma but a reduction in the volume available for the distribution of T-1824. They concluded that part of the circulation was closed off as a result of this stress and thus plasma (D'Amato & Hegnauer, 1953) or blood (Rodbard *et al.* 1951) was locked away. This explanation would account for their evidence, inasmuch as they used successive injections of the dye; the first was a control and subsequent injections were given during cold stress. In our case the disappearance of a single dye injection was observed and the 'shutting off' of a part of the circulatory system would not account for the changes observed in the rate of disappearance of the dye that occurred after cold immersion. It might be argued that in the case of the work cited above (Rodbard *et al.* 1951; D'Amato & Hegnauer, 1953) that changes in the endogenous optical properties of plasma were responsible for the observed increase in dye density. However, such an argument does not hold when the dye concentration has been ascertained by quantitative extraction as in our experiments. To explain the failure of the proteins to reflect, by increased concentration, egress of fluid, we offer two possible explanations. There may be, as a temporary result of the increased metabolic rate accompanying cold stress (Bird, Brown, Lennox & Semple, unpublished), a change in the quality of the circulating protein. Wasserman & Mayerson (1951, 1952) have shown that in normal dogs the globulin fractions are metabolized at about twice the rate of the albumin fraction. It is thus possible that on cold immersion there would occur a preferential reduction in the globulin fraction. Semple (1955) has reported that after haemorrhage and dextran infusion there is at first preferential reduction in the globulin and fibrinogen fractions of dog plasma. In the case of fibrinogen there is evidence of a specific reaction with the dextran (Ricketts, 1952) but there is no such evidence for globulins. It is true that stress is usually followed by an increase in globulin fractions (Selye, 1950) but this does not preclude the possibility that there occurs with cold a temporary and preferential increase in globulin catabolism, a marked decrease in the rate of return of protein to the circulation, and a significant fluid loss to the interstitial spaces. An alternative possibility is that the dye-labelled albumin has a different metabolic behaviour from albumin not so labelled. The results of a number of investigations indicate that the *in vivo* activity of the albumin molecule is affected by either chemical or radioactive 'labels'. Sear, Allen & Gregersen (1953) have shown that in dogs the disappearance rate of T-1824 from plasma is very much greater than is the disappearance rate of ^{131}I. Similarly, Masouredis & Beeckmans (1955) found that albumin labelled with ^{131}I leaves the plasma at more than twice the rate of ^{14}C-labelled albumin, and Margen & Tarver (1956) report large differences between the metabolic activities of ^{131}I and ^{35}S-labelled albumins. In cold stress experiments, then, the possibility that the dye-tagged albumin did not share proportionately in the increased metabolism cannot be excluded. If this were the case, the artificial protein fraction has reflected the loss of vascular fluid, whereas the unlabelled albumin and the other protein fractions have not. The mechanisms behind the failure of the total protein concentration of plasma to reflect the fluid loss that accompanies acute cold stress are in need of further study.

SUMMARY

1. Plasma volumes, haematocrit values and plasma proteins were measured under control conditions in a group of adult male Eskimos and in a group of medical students in Kingston, Ontario. Changes in these parameters that resulted from immersion of one arm in a water-bath at 5° C for one hour were then observed, and final observations were made during the forty minutes that followed the end of arm immersion.

2. The Eskimo plasma volumes were significantly greater relative to body weight, surface area and body fat content than those of the Kingston group, and resulted in high blood volumes. The authors believe the high blood and plasma volumes of Eskimos to be a feature of cold acclimatization. Eskimo haematocrit values were lower than those of the Kingston group, but there was no significant difference between the two groups in respect to red cell volumes. Protein concentrations in Eskimo plasma were significantly higher than those of the Kingston group.

3. Immersion resulted in decreased plasma volumes and increased haematocrit values. These changes were somewhat greater and occurred more rapidly in the Eskimo group, but the differences between the two groups in these respects were not significant. Immersion resulted in no significant changes in total red cell volumes.

4. After the end of the immersion the haematocrit values returned more quickly to control levels in the Eskimo group than in the white group.

5. Plasma protein concentrations remained essentially constant in spite of the reductions in vascular fluid that were indicated by changes in T-1824 concentrations and increases in haematocrit readings during immersion. These observations are discussed.

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HEAT TRANSFER MEASUREMENT IN LIVING SKIN

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Measurements of temperature gradients and heat flow in the superficial skin layers are of importance in the study of body heat regulation. Though skin seems accessible, difficulty is found in making temperature measurements. Skin is very thin; often epidermis is less than 0·05 mm thick on the limb surfaces, there are large changes in skin properties in a small depth of tissue and rapid changes in vessel size and blood flow may follow minute damage. For these reasons an exploring thermocouple must be small compared with epidermis and should cause little physical change on insertion or local heat flow will be affected.

It is difficult to make couples to this standard from easily obtained materials, since commercial wires are usually from 0·01 mm upwards in diameter and the finer wires are too fragile and flexible to use without support. Usually they are platinum alloys with low thermo-electric e.m.f. The smallest couple which could be made is thus 0·02 mm in diameter, and larger if supported strongly enough for insertion into skin.

Previous work (Bazett & McGlone 1927; Bazett, 1941, 1949; Mendelsohn, 1936), while demonstrating the presence and general form of the temperature gradients, has suffered inevitably from these difficulties. Needle-mounted couples of 0·3–0·8 mm diameter had to be used. Conduction stem errors large enough to be calculated and of a similar order to the differences being measured have had to be allowed for in some cases. This calculation can only be done approximately or empirically, as the physical system is too complex to allow rigorous treatment. (It involves two media, both non-homogeneous, one of which may vary in time. These media interact in three dimensions.) Also, although stem errors near the surface may be allowed for, those occurring in deeper tissues, where the gradient is steep or changing in direction, cannot be estimated.

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Attempts have been made to measure temperatures in the most superficial 2 mm of skin with couples 0·3 mm thick with soldered junctions of the same order of length (Bazett & McGlone, 1927; Mendelsohn, 1936). Though every care has been taken to minimize skin damage and the gradients obtained were shown to differ in degree from those where damage was great, it seems likely that they must represent somewhat inflamed skin. The couples used had diameters many times those of the largest vessels in the outer layers. Another difficulty has been the estimation of skin heat flow exactly at the site and time of estimation of a temperature gradient. Pennes (1948) gives evidence from his own and other work of the great variation of surface temperature from point to point over small areas of skin. This has been fully confirmed by the method to be described later in this paper. For this reason, and also because skin temperature locally may change with time in a subject exposed for a long period to a constant environment, it is necessary to measure heat flow at the site of thermocouple entry. The couple should therefore not be inserted obliquely, as is sometimes done to avoid stem errors. Frequent readings of skin heat flow need to be taken during an experiment, for if it changes between gradient readings the apparent form of the gradient will be affected.

An attempt has been made to overcome these difficulties by devising a very small thermocouple surrounded by a heat-flow measuring device. This paper comprises an account of the construction and performance of this system.

METHODS

The thermocouple

The thermocouple (Vere, 1955) is designed to be very small yet rigid, with a stem conduction error smaller than the experimental error from other causes. It can be passed into a hair follicle, or a large sweat or sebaceous gland. Couples of 0·05–0·03 mm diameter may be passed into skin along the track of a puncture by a glass fibre of the same size causing relatively little damage.

General structure. The general arrangement of the couple is shown diagrammatically in Fig. 1. The couple is formed between a bismuth-antimony alloy in a fine glass capillary and an outer coating over this of Wood's metal. The exploring junction lies at the tip of the capillary. Thermo-electric currents are led away through copper wires joined respectively to the two alloys at the upper, broad end of the glass capillary. These junctions are surrounded by a constant-temperature bath and constitute the reference junctions. All metal surfaces are insulated electrically with a thin layer of rubber.

Method of construction. New and carefully cleaned soda glass tubing of about 6 mm diameter and 0·8 mm wall is drawn down to 1–2 mm diameter. Its composition seems to be critical, as good results have been obtained only with certain types of glass (Osram–G.E.C. 'Wembley' X8 soda glass). A little of one of the Hutchin's alloys (Bi 97, Sb3 parts; Firestone, 1930) is melted in a Pyrex boiling tube. While the drawn-down glass capillary is still hot some of this bismuth alloy is drawn into it with a rubber suction bulb. Before it cools the metal-in-glass capillary so made is further drawn down in stages till it is about 0·3 mm diameter. It can be heated to bright redness without damage. When it is 0·5 mm or less in diameter it may be allowed to cool with only a few small cracks occurring in the glass. Larger capillaries shatter on cooling. A few small cracks are unimportant; they seal over during the next stage. A fine tinned copper wire is melted into the alloy at one end of a suitable piece of capillary, which has been filled with alloy in the way described. The capillary is then heated, about 2 cm from the seal to the copper wire, in a micro-burner with a minute flame. When bright red hot the capillary is quickly pulled out into a very fine capillary, the broad end near the copper being returned quickly to the flame after this manœuvre and passed through the flame again rapidly towards the copper wire. In this way the alloy solidifies last at the broad end and forces due to solidification of the alloy do not crack the glass where the capillary diameter varies sharply.

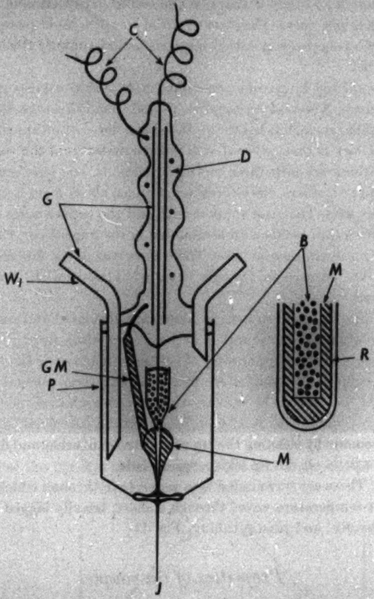

Fig. 1. Diagram of the general arrangement of the thermocouple. The rubber coat covering structures in the reference junction bath is omitted for simplicity. Inset, tip of couple enlarged. *B*, bismuth alloy in glass capillary; *C*, copper wires; *D*, 'Durofix' cement; *G*, glass tubes; *GM*, glass coated with Wood's metal; *J*, exploring junction; *M*, Wood's metal; *P*, paper reference-junction box; *R*, rubber; W_I, water inlet.

The fine end of the capillary is then placed in a drop of melted Wood's metal (m.p. 70° C) on a heated light alloy block. This block (2 × 1 × 0·5 cm) is surrounded by a copper shim apron to ensure even heating and avoid contact of hot fumes with the capillary. The capillary is broken off in its finest part, under the melt (to avoid exposure to air) and about 2 cm from its broader end. It is then pushed through the melt till its narrow end protrudes and its stem is coated with Wood's metal nearly up to the broad end but not over it. This is because small cracks in the glass here short-circuit the couple when the melt is carried over them. The exploring thermal junction is made at the capillary tip between the inner alloy and the Wood's metal coat. If a junction is not formed at once it is due to a fault in the alloy core. A little more capillary is then broken off in the melt and so on till a good junction is made. Oxide quickly forms on the melt, which must be cleaned frequently by drawing a razor blade across its surface.

The formation of the junction is simplified by connecting a microammeter between the light alloy block and the copper wire fused into the capillary. Formation of a good junction is shown on the meter by the large thermal current produced. Good junctions are not formed if the light alloy block is dirty or too cool, when crystals can be seen in the melt. If it is too hot the melt will not coat the glass. It should be just hot enough to melt all the crystals and not more. When a junction forms, the couple should be finished quickly, as sometimes if it is kept hot the resistance rises slowly and the junction becomes unstable. The capillary is pushed once or twice through the melt to coat it thoroughly and then withdrawn and cooled. Inspection in a good light shows any small irregularities as bright spots. They are removed by a few rapid passes through the melt. It seems likely that sputtering or evaporation methods would simplify this stage greatly, but they have not yet been tried.

The couples are completed by passing the copper wire inside a glass supporting tube (about 3 mm external diameter). A second copper wire, wound round this tube, is pushed into the end of a fine empty glass capillary which is led to the Wood's metal coat on the couple capillary (Fig. 1). This empty glass capillary is then covered with Wood's metal from the coat on the couple to the second copper wire. There are now three metallic junctions, one between the two alloys at the tip of the capillary and two others, the reference junctions, about 4 cm from the tip, each between one alloy and a copper wire. The glass support tube and the copper wires are coated and secured with 'Durofix' cement. A thin Perspex fin is mounted in the cement covering the glass supporting tube, to be gripped by a micro-manipulator. The connexions of the fine copper wires to the leads of a mercury switch are encased in a single block of 'Plasticine' to protect them from small temperature differences.

The whole couple up to the glass support is electrically insulated and water-proofed by dipping once in thin rubber solution. If the couple is held point up after dipping, only a very thin coat is left on the tip. A second heavier application of rubber is made over the reference junctions and broader parts of the couple. The couples are inspected and their diameters measured under the microscope. The metal coat should be smooth with only small variations in diameter (Fig. 2).

Couples last for many months if well made. They can be completed in 15 min with practice. Broken ones may be remade by cleaning the tip with petroleum ether and dipping again in Wood's metal after breaking a little off the tip with a razor blade.

Reference junctions. These are surrounded by a water-bath through which a steady flow of water is led from a constant-temperature vessel through a short, heavily lagged rubber tube. The bath is made of paper, 'Durofix' and plastic tubing (Fig. 1).

Properties of the couples

(1) They are very small, being easy to make over the range 0·05–0·005 mm in diameter.

(2) The thermo-electric e.m.f. is high (about 58 μV/° C). This overcomes in part the disadvantage of high internal resistance of small couples.

(3) The calibration (Fig. 3*a*, *b*) is almost linear over the range 10–40° C. Lower temperatures have not been explored. Above 45° C the junction may become unstable. Fig. 3*a* shows results over a wide range of temperature with a 400 Ω series resistor in circuit. Fig. 3*b* shows results over a smaller temperature range without a series resistor.

The couples are calibrated by dipping their tips into a glycerol bath in a large brass block containing a mercury-in-glass thermometer reading to 0·05° C directly and to 0·01° C by interpolation. The block is placed in stirred water in a Dewar flask. The normal practice of immersing a good length of the couple in the medium cannot be followed here. Great instability results from air convecting from the glycerol surface up towards the reference junctions. This is not a conduction stem error. It is completely removed by covering the exposed stem with cotton-wool. In

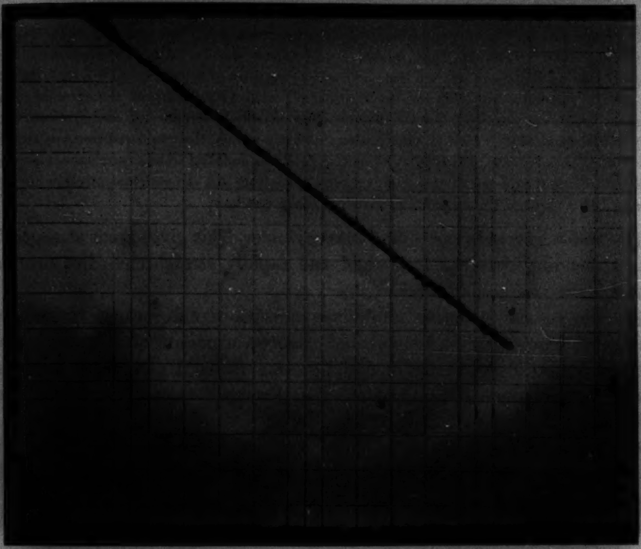

Fig. 2. Photograph of the tip of a thermocouple 0·006 mm diameter mounted on a red cell counting graticule.

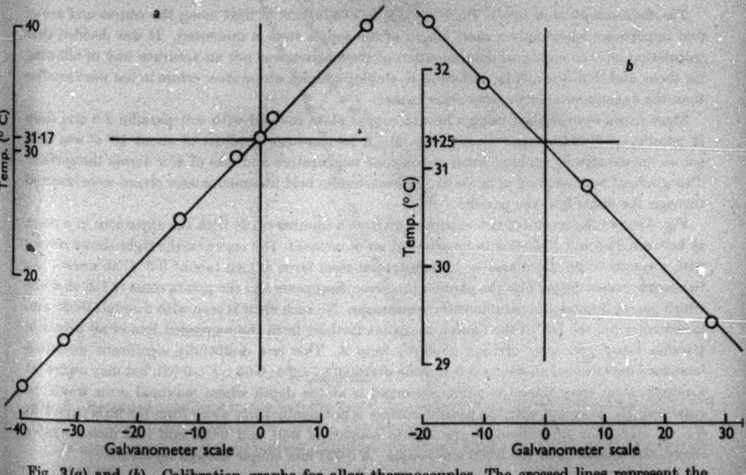

Fig. 3(*a*) and (*b*). Calibration graphs for alloy thermocouples. The crossed lines represent the temperature of the reference junction bath and the galvanometer zero. The conditions of the experiments are given in the text.

use on skin this difficulty is overcome by surrounding the lower part of the stem with a water 'flow-box' to be described.

The apparatus used in this and all the other experiments described was as follows. The galvanometer was a 'Cambridge Spot' model. Its sensitivity was 7·2 μA for 100-scale division deflexion. One scale division was 1·6 mm. It could be read by interpolation to 0·1 scale division. Its internal resistance was 27 Ω and the suspension period 2·6 sec. Though the external critical damping resistance was 88 Ω, lower resistances were usually in circuit and readings were taken only in the steady state. All lead wires ran to a mercury switch, their soldered contacts all being in the same paraffin bath. The reference junction bath was supplied from a constant-temperature bath controlling the temperature to 0·01° C; water came from this at a constant pressure head and returned to it through a filter pump powered by compressed air.

(4) The structure is based on a glass cylinder. This gives great strength for lateral bending stresses. It is rigid and rapidly straightens after bending, which is not true of fine wires.

(5) The coaxial structure minimizes parasitic e.m.f. because surrounding fluids can reach only one metal and the two elements of the couple pass through almost identical electrical and thermal fields. However, an insulating coat is essential, as large contact potentials develop between Wood's metal and solutions.

(6) The exploring junction is planar and normal to the axis. There is no junction error such as occurs with long soldered junctions of wire couples where the exact site of generation of potential is uncertain.

(7) The thermal conductivities of all the couple materials are far lower than those of common metals, the Wood's alloy, glass and Hutchin's alloy having conductivity of 0·032, 0·002 and 0·02 cal/cm.° C.sec respectively. This, together with the very small cross-section, minimizes stem conduction error.

The thermocouple stem errors. These are due to conduction of heat along the couple and are of first importance where only a short length of the couple stem is immersed. It was decided that calculation or even empirical determination of these errors was not an accurate way of allowing for them, and that it would be preferable to employ couples whose stem errors in use were smaller than the experimental error from other causes.

Stem errors were studied using a heated copper plate covered with soft paraffin 2·6 mm deep (k paraffin = 0·00044 cal/cm.° C.sec) (Fig. 4). A temperature gradient of about 10° C was held across the paraffin by running water at constant temperature and rate of flow across the surface. This gradient was assumed to be linear. Thermocouples held in a micrometer clamp were inserted through the water into the paraffin.

Fig. 5 shows the results of two experiments from a number made with the apparatus in a room at between 24·5 and 25·5° C with no artificial air movement. The curve on the right shows results with a couple 0·090 mm diameter. A substantial stem error is seen (about 0·2° C at worst). As the couple passes deeper into the paraffin this error disappears and the points come to fall on a line which passes through the paraffin surface intercept. No such error is seen with a couple 0·025 mm in diameter (on the left of the figure), the point farthest from the regression line of all points in paraffin being 0·36 scale division (0·06° C) from it. This is a doubtfully significant deviation (standard deviation of all points is 0·15 scale division, $t_{22} = 2{\cdot}4$; $0{\cdot}05 > P > 0{\cdot}02$), but may represent a minute stem error since the point concerned is at the depth where maximal error would be expected. The intercept with the paraffin surface is not significantly away from the lines ($t_{22} = 1{\cdot}3$; $0{\cdot}5 > P > 0{\cdot}2$). In other similar experiments couples of 0·07 and 0·050 mm had demonstrable errors, but smaller than the 0·090 mm couple. A 0·022 mm couple showed no error.

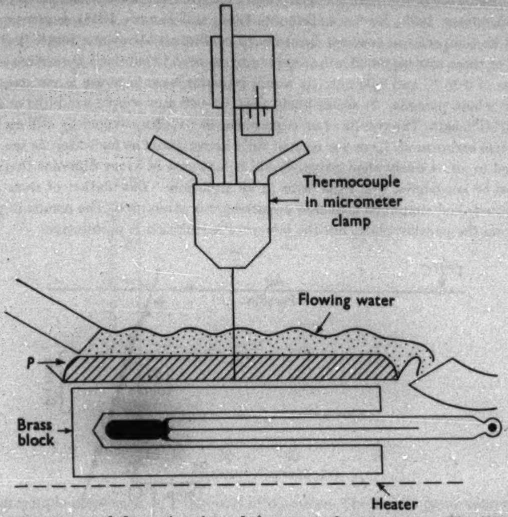
Thermocouple
in micrometer
clamp
Flowing water
P
Brass
block
Heater

Fig. 4. **Apparatus arranged for estimation of thermocouple stem errors. The thermocouple is lowered into a layer of paraffin, *P* (on copper plate), across which a constant temperature gradient is held; see text.**

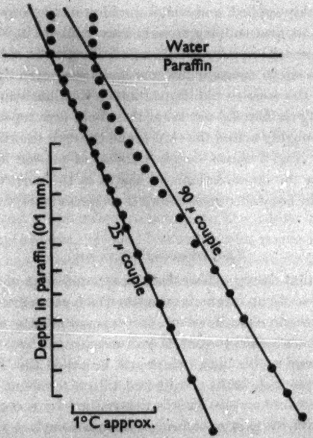

Fig. 5. **Recorded gradients in the paraffin plate. The ordinates represent depths in paraffin and the abscissae galvanometer deflexions. The temperature scale indicating the order of errors is approximate, as the calibration of the galvanometer differed a little for the two thermocouples.**

These conditions are much more stringent than those for skin, as its conductivity (discussed by Hardy & Soderstrom, 1938; Burton & Edholm, 1955; and Pennes, 1948), is greater than that of paraffin and its temperature gradient in ordinary conditions about one tenth that of these experiments. For these reasons 0·025 mm couples may be used in skin and their stem errors ignored for accuracies of 0·05 °C and 0·05 mm. It would probably be safe to use larger ones but this has been avoided where possible. It seems likely that a 0·040 mm couple would have a worst error of about 0·05° C in skin. The results of an experiment in paraffin covered by still air are shown in Fig. 6. The stem errors in air have not been as fully investigated as for water, as the couples were not to be used in air. Clearly stem errors should be assessed in every different thermal case and no couple can be considered free from error in all situations. Calculation of stem error by 'relaxation' methods and simplified heat-flow equations was attempted. The results in general agree with those from the paraffin plate, but the labour of calculation is prohibitive.

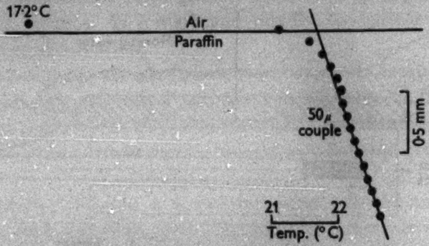

Fig. 6. Estimation of thermocouple stem errors by progressive immersion in paraffin. Recorded gradient with air above the paraffin plate.

The insulating coat. The rubber coat is very thin and seems to interfere little with thermal performance. Were it more thickly applied, however, it would probably worsen stem errors. The rubber is not entirely satisfactory as firm rubbing on skin may damage it, lowering the resistance. It was, however, found to be much superior to 'Durofix', Perspex in chloroform or polystyrene in ethyl acetate. It was difficult to measure the coat resistance directly, but Wood's metal rods coated in the same way as the couples and dipped into 1% saline solution had a coat resistance of about 7×10^5 Ω. Touching on skin did not lower this. Very firm repeated pressure lowered it to about 10^5 Ω and scraping roughly across the skin broke through the rubber completely.

Thermocouple resistance. Fig. 7 shows couple resistances plotted against diameters. The resistance depends mainly on the cross-section and length of the narrowest part, the latter being approximately constant. The resistance scale shown is reciprocal and the diameter scale represents a square function.

The thermocouples in use

It has been pointed out that during calibration the exposed stem of the thermocouple must be surrounded by cotton-wool to damp down air currents which cause instability. In the determination of temperature gradients in skin, however, the exposed stem is surrounded by a constant-temperature water-bath which is used to control and measure the heat loss from the skin surface. The temperature of the water in this bath (which will be called the 'flow-box' to distinguish it from the couple reference-junction bath) is adjusted till on touching the skin with the box no change in local skin temperature occurs. This is determined by a copper–constantan couple of Y shape lightly stretched over the skin so as just to indent its surface at the site of contact of the bath. This couple is similar to the design described by Bazett & McGlone (1927) and discussed by Pennes (1948) and Mendelsohn (1936). If the skin surface temperature remains the same it seems likely that no thermal change will occur in the tissue beneath it.

The flow-box is formed from a cylindical Perspex box (approx. dimensions: wall 1, external diam. 18, height 15 mm). Into the base is inserted a Hatfield-Turner heat-flow disk (Hatfield & Wilkins, 1950; Hatfield, 1950; Holti, 1955). Box and disk are divided along a vertical diameter into symmetrical halves, each half of the box holding one half of the disk: the thermo-electric e.m.f. from the latter is led from one half only.

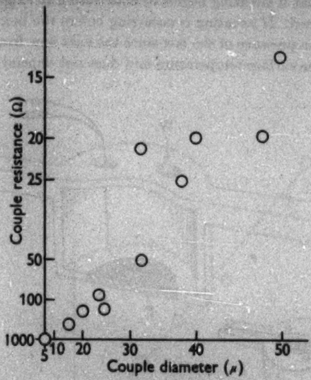

Fig. 7. Thermocouple resistance as a function of diameter. The lowest point represents 7000 Ω and 0·005 mm diameter. The resistance scale is reciprocal and the diameter scale represents a square function.

Each half of the box is provided with two arms projecting horizontally at the level of the upper end in the plane of division (Fig. 8*a*), for clamping the box to a Perspex plate on which the micromanipulator is mounted (Fig. 11). At one extremity the corresponding arms of each half are hinged together (Fig. 8*b*): the other pair of arms can be drawn together by a screw (not shown), thus approximating the two halves of the box. The slit in the centre of the lid is widened into a lanceolate aperture, on each side of which are two openings for the circulation of water. The inner wall of each half of the box is made of rubber hydrochloride 0·0015 in. (0·038 mm) thick. The two halves are held on the Perspex plate which forms the base of the micromanipulator, so as to embrace the stem of the thermocouple after it has been passed into the skin: the screw then approximates the two halves till they almost touch (Fig. 8*c*).

Water from the couple reference-junction bath is led through the box, holding it at constant temperature very close to that of the reference junctions. The rubber walls touch the stem of the couple over a large area. This ensures that heat loss up the stem occurs to a sink of constant known temperature, large size and known distance from the tip. Three thermocouples are used with the box. They are the heat-flow disk itself, a fine copper–constantan junction, calibrated before incorporation and placed in the water, and the movable Y-shaped junction already mentioned. It was found that a couple fixed under the box gave erroneous readings of skin temperature. This is discussed later. The flow-box couple was of 44 s.w.g. copper and 36-gauge constantan thinned for its last inch to 44 s.w.g. in nitric acid. The skin Y couple was of 32 s.w.g. wires. The leads were short and unjointed. The reference junctions were in a tube of liquid paraffin in the constant-temperature bath.

The advantages of the flow box are thought to be as follows:

(1) The heat-flow disk gives a most sensitive measure of heat flow from the skin at the site of thermocouple puncture.

(2) By covering the surface, air-flow errors are removed.

(3) Heat flow up the couple may be stabilized and estimated.

(4) Local conditions are stabilized for a time, allowing more accurate thermal gradients to be found.

(5) All the parameters may be found for direct measurement of the thermal conductivity of skin layers.

The disadvantage is that the presence of the box interferes with radiant heat exchange and evaporation from the skin, so that if sweating begins or ends during an experiment a false idea of total skin heat loss will be obtained. If sweating is occurring before the box is applied, the total heat loss will be unaffected by the presence of the box since the heat flow from the deep tissues to the surface is determined by the surface temperature and does not depend on the nature of the heat exchange at the surface.

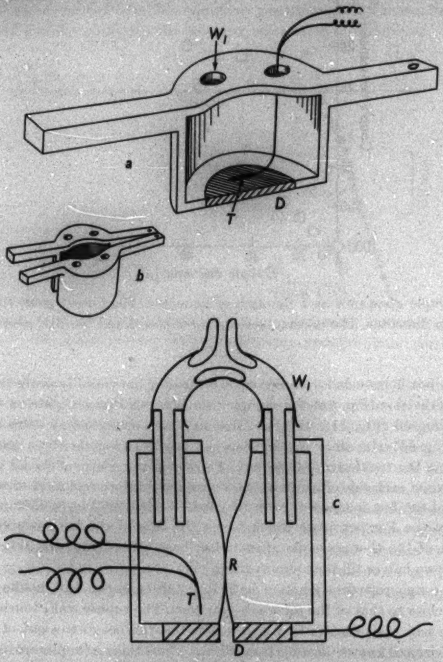

Fig. 8. The flow-box. *a*: one half of flow-box, with rubber membrane of inner wall removed; W_I, water inlet; *D*, heat-flow disk; *T*, flow-box water thermocouple, just above disk—in practice the wires to the water thermocouple lie in the water for over 2 cm of their length. *b*: the two halves assembled for use. *c*: section across two halves of box; *R*, rubber hydrochloride membrane.

The calibration of the flow-box was made in two ways. First the thermocouples were calibrated with known temperature differences between the box and its surroundings, and secondly with known heat flows through its base. The box was placed with the heat-flow disk just on the surface of a well stirred constant-temperature water-bath. The graph (Fig. 9) shows the calibration results with water at constant temperature flowing through the box. The regression coefficient of galvanometer readings on temperature difference across the plate was 58·7. On the stirred bath the plate is less stable than on skin. However, the standard deviation of the readings from the regression line shown is 0·045° C. On skin the standard deviation of repeated results over 10 min is of the order of 0·01° C. The regression coefficient for the calibration of the copper–constantan couple in the flow-box water was 11·9 scale divisions/° C. The standard deviation of one reading was 0·03° C under calibration conditions.

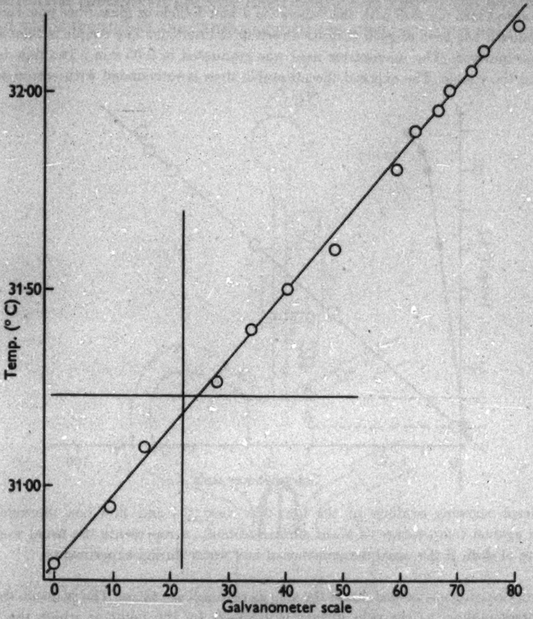

Fig. 9. Heat-flow disk calibration graph for differences of temperature across the disk. The crossed lines represent the temperature of the water in the flow-box and the zero of the short-circuited galvanometer.

Fig. 10 shows a similar graph but with temperatures recorded within the flow-box water. The box temperature is affected by heat flow through the base to a measurable amount only when the heat flow is large compared with that to be expected from skin in ordinary circumstances. (The results are offset a little from the origin owing to cooling of the water reaching the box after leaving the constant-temperature bath at 28·50° C.) Normally the water is only about 0·5° C below skin temperature. In spite of this rise in cooling water temperature it will be seen that there is no measurable non-linearity of heat-flow disk calibration over a range even beyond its normal use.

To calibrate the box with known heat flows a second Hatfield heat-flow disk was used. This was mounted to coincide with the disk in the base of the box. A layer of thin cloth soaked in glycerol made good thermal contact between the disks. The water flowing through the box was held at a steady temperature while readings were taken with the heat-flow disk of known calibration placed in circuit with the unknown disk and the galvanometer. The known disk was turned over during each experiment so that for each temperature setting of the box a sum and a difference of the e.m.f. produced by the disks was obtained. From these and the galvanometer zero the ratio was obtained between the currents from the two disks for a given heat flow identical for both. The calibration of the box disk was 4·23 μV for a heat flow of 10^{-4} cal/cm^2.sec.

The full assembly for a skin experiment is shown in Fig. 11. After the box temperature has been set, the Perspex plate is strapped in position on the skin with adhesive plaster. Then the thermocouple is inserted into the skin with the fingers via a hair follicle or glass-fibre puncture after the micromanipulator has been aligned with its direction of insertion. The couple is then clamped in the micromanipulator. (The micrometer used was graduated in 0·05 mm.) The flow-box is then placed round the couple. The exposed thermocouple stem is surrounded with cotton-wool above the flow-box. The couple is racked out of the skin as readings are taken. The point on the recorded gradient corresponding to the skin surface is assessed by the point at which the previously measured skin temperature occurs, by direct inspection of the site of the couple tip after the experiment, racking it down till it coincides with the lower edge of the box, and by inspection of the shape of the gradient. The part of the gradient produced when the tip is between the halves of the heat-flow disk is linear; the part when the tip is in skin has a characteristic curve. There is a small gap between the two parts in the curve (Figs. 15, 16).

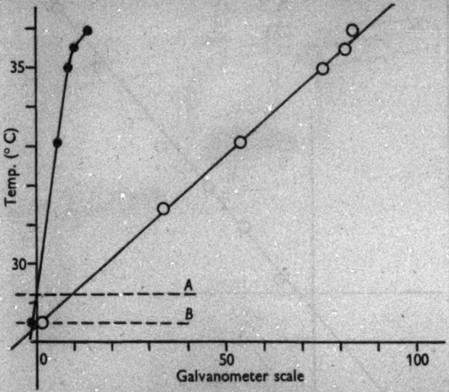

Fig. 10. Graph showing readings of the heat-flow disk (○) and flow-box thermocouple (●) plotted against temperature of lower surface of box. *A* represents the usual working temperature of skin, *B* the usual temperature of box water during experiments.

RESULTS AND DISCUSSION

The performance of the apparatus

All the experiments have been made in a room with wet- and dry-bulb temperatures controlled to ± 0·6° C and a constant air movement of about 50 ft./min. The wall temperature was quite variable, but changes occurred very slowly; they may therefore cause differences between but not within experiments.

With the apparatus on the skin it seemed likely that major contributions to error would be made by room climate change, subject–instrument errors and a residual error due to galvanometer reading and small circuit e.m.f. due to unavoidable physical effects. A simple semifactorial experiment was attempted to study this complex error. In addition to the skin Y thermocouple, a fourth thermocouple was fixed below one half of the box with 2 cm of its lead wires wound in the same plane to see whether this would give better estimates of local skin temperature. Reference junctions were in the water-bath, whose temperature could be read to 0·01° C and was shown never to vary by more than 0·05° C and seldom more than 0·02° C during an experiment. Equal numbers (fourteen) of readings were taken from the heat-flow disk and the copper–constantan couples at intervals during a measured time. For readings on skin the box was adjusted so as not to alter local skin temperature. The site was front of thigh and the conditions identical with the skin gradient experiments except in the mounting of the box on the thigh—the box rested lightly on the skin. During measurement of temperature gradient

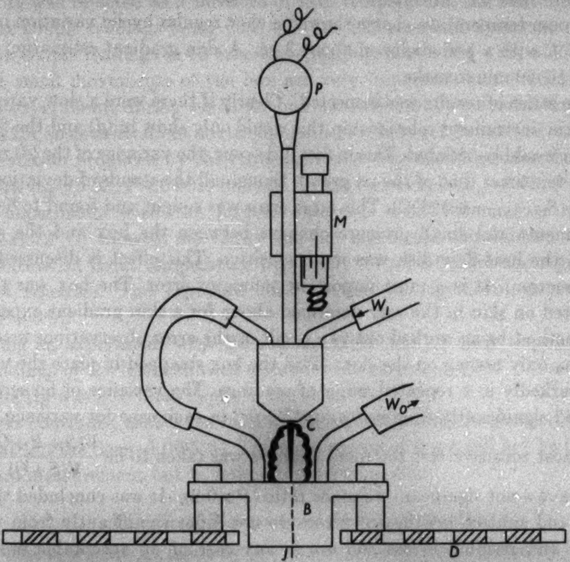

Fig. 11. The full thermocouple assembly. *P*, 'Plasticine' round soldered junctions to thermocouple leads; *M*, micrometer clamp holding thermocouple; W_I, W_O water inlet and outlet; *C*, cotton-wool round stem of thermocouple; *B*, flow-box; *D*, drilled Perspex plate; *J*, exploring junction of thermocouple, in skin.

in skin the box was more securely mounted, a change of method suggested by these error experiments. Four series of readings were made.

Series	Purpose
(a) Apparatus just on the surface of water in constant temperature bath. Results taken over 2 min.	To eliminate skin (S) and room (R) changes leaving other errors (O)
(b) As (a) but over 20 min.	To eliminate (S) but not (R) changes leaving (R + O)
(c) Resting on skin, 2 min.	To eliminate (R) but not (S) leaving (S + O)
(d) On skin, 20 min.	To include all errors (S + R + O).

The room temperature charts showed a slow regular cyclic variation of about $\pm 0{\cdot}3°$ C with a periodicity of about 3 hr. A skin gradient estimation usually takes 10–20 min to make.

The series of results was inspected. Clearly if there were a slow variation in the skin–instrument relationship this would only show in (*d*) and the factorial design would be vitiated. This in fact did occur, the variance of the (*d*) readings being ten times that of the (*c*) group, though all the standard deviations were small ($S_{(S+R+O)} = 0{\cdot}026°$ C). This large error was sought and found to be due to movements and small pressure changes between the box and the skin, to which the heat-flow disk was most sensitive. This effect is discussed in the next section. It is a most important source of error. The box was then remounted on skin in the way described above for a skin gradient experiment, this method being worked out as a result of the error observations made with the box only resting on the skin. With the box strapped in place the variance fell markedly in a repeated series of readings. The variance of no series now differed significantly from series (*a*) ($P > 0{\cdot}2$ in each case for variance ratios). The most sensitive test for room changes was taken to be $\dfrac{V(S+R+O)}{V(S+O)}$. This now gave a not significant variance ratio ($P > 0{\cdot}2$). It was concluded that the room and subject–instrument errors do not differ significantly from the observer and residual errors and are in any case of an acceptable order, the standard deviation of one heat-flow disk reading for a long duration experiment on skin being about $0{\cdot}01°$ C provided the site and pressure on the skin are fixed.

The error of the couple in the flow-box was of a similar order. The skin temperature couple fixed beneath the box gave variable readings which tallied badly with heat-flow disk results. Its readings showed a steady change during the experiment. Longer series of readings were made to decide whether the plate and box water combination of couples or the skin couple fixed beneath the box gave the better estimate of skin temperature. As the box water couple does not change significantly, Hatfield disk readings alone were used. Multiple regressions on room-temperature change and reading order in time of both the 'skin' couple and the heat-flow disk were made. These showed that while the Hatfield disk was independent of both ($0{\cdot}3 > P > 0{\cdot}2$ in each case) the skin couple was independent of room temperature only ($0 \cdot 7 > P > 0 \cdot 5$). It showed a very clear dependence on the order of making readings ($P = 0 \cdot 001$). Thus the readings of a skin temperature couple fixed under the box drift slowly with time and depend on no other parameter. This appeared to be due to a progressive indentation of the epidermis by the couple, which can be seen for some time when it is lifted from the skin. In this way it shares to a variable extent the temperatures of the skin and box temperatures during the experiment. It was rejected as a measure of skin temperature, the heat-flow disk and box couple readings being used instead. The standard deviation of disk and box couple readings in 55 successive readings was 0·014° C in each case.

That small movements of the box are very important was confirmed by further experiments during which the box was deliberately moved a very little on the skin, so as to lift it but not to change its site, between two sets of readings. The deviation produced by the movement was very significant for the heat-flow disk ($t_{16} = 5 \cdot 28$, $P < 0 \cdot 001$) but not for the box couple ($t_{16} = 0 \cdot 08$, $P > 0 \cdot 9$).

An experiment with the largest observed room temperature change (21·45–21·72° C) showed the slight but not significant dependence of box temperature on room temperature ($t_{10} = 1 \cdot 23$, $0 \cdot 3 > P > 0 \cdot 2$ for the regression sum of squares). The heat-flow disk showed no significant dependence ($0 \cdot 7 > P > 0 \cdot 5$).

During an experiment with the full assembly and an alloy thermocouple the readings of the latter showed a standard deviation of 0·026° C for 16 min while its tip was at a fixed depth in the skin.

It was concluded that when used in the way described the method had an error within the desired 0·05° C. The main source of error will not be in temperature measurement but in estimating depth in skin.

The effects of pressure of the box on the skin

The effects of pressure made by the box on the skin were studied by loading it with small weights. These were placed on it for 15 sec in each case, with a 15 sec unloaded period between loads. Fig. 12 shows the readings of the heat-flow disk during the experiment. The area of the box base was 250 mm^2. Thus the box loadings equivalent to capillary, diastolic and systolic pressures would occur roughly at 85, 270 and 410 g respectively. Fig. 13 shows the effect of firm digital pressure on the box. In these experiments reactive hyperaemia developed progressively as the pressure increased, being visible as a red colour when the box was lifted and persisting for a prolonged period afterwards. The colour remained red and did not fade after the experiment for many minutes. The temperature given by the hyperaemic skin after maximal pressures was in each case equal to or a little higher than that recorded during the pressure if the pressure was short in duration but very heavy. More prolonged heavy pressure resulted in progressive cooling during compression.

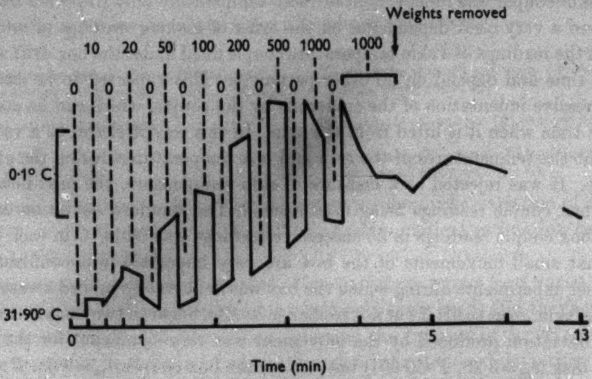

Fig. 12. The effect of weighting the flow-box on recorded skin surface temperature. The figures above the diagram represent grams weight applied to the box.

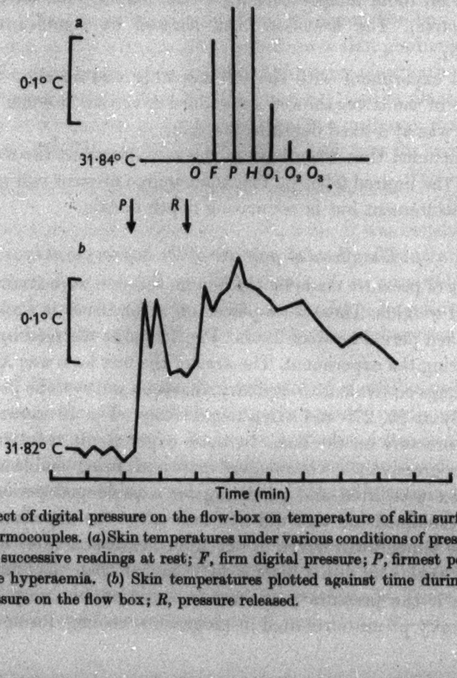

Fig. 13. The effect of digital pressure on the flow-box on temperature of skin surface recorded by the box thermocouples. (*a*) Skin temperatures under various conditions of pressure: *O*, resting; O_1, O_2, O_3, successive readings at rest; *F*, firm digital pressure; *P*, firmest possible pressure; *H*, reactive hyperaemia. (*b*) Skin temperatures plotted against time during and following digital pressure on the flow box; *R*, pressure released.

While prolonged very heavy pressure may empty all vessels locally with a consequent fall in temperature, it may be that pressure heavy enough to empty veins but not arteries or a short very heavy pressure gives a skin temperature elevation of the highest order found by Lewis (1927) in reactive hyperaemia but not as high as that due to full arteriolar dilatation with maximal skin blood flow. It is possible that in the former case the skin temperature may approximate to that of local arteriolar blood. This is uncertain, but the results do show the very large effects of pressure, even though quite light, on local skin temperature. For these reasons frequent heat-flow readings should be made while estimating a skin thermal gradient, for a change in the site or pressure of the apparatus may cause a discontinuity in the gradient which might be attributed to other causes such as local vascular anatomy. A gradient in a hair follicle showing this effect, due to a small change in position of the box, is shown in Fig. 14. Visible veins were not included in the area beneath the box in skin heat-flow experiments as their cooling or heating effects are most variable.

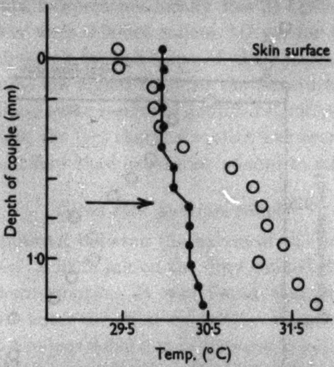

Fig. 14. A skin thermal gradient during which movement of the flow-box occurred. ○, thermocouple readings; ●, coincident heat-flow disk readings. The arrow indicates the depth of the thermocouple at which the box movement occurred. Readings were made at regular intervals of depth, in order, from the point of deepest insertion.

Thermal gradients in hair follicles

It was found easy to insert a thermocouple into a hair follicle, after removal of a hair from the skin on the front of the thigh, although the follicle often entered at an angle necessitating tilting of the pillar of the thermocouple clamp. A typical result is shown in Fig. 15. Depths are measured along the axis of the follicle which lay at 40° to the normal. It soon became clear when skin puncture experiments were made that the follicle gradients differed from the direct puncture results. The form of the follicle gradients differs from that of skin puncture measurements and would be affected by the known conical shape of follicles and the specialized vascular plexus surrounding them (Johnston & Whillis, 1949). This may account for the absence of large temperature peaks and marked changes of gradient found by Bazett (1949) in punctured skin and ascribed by him to the pattern of blood distribution in the dermis. However, small temperature peaks can be seen at 0·3 and 0·55 mm depths.

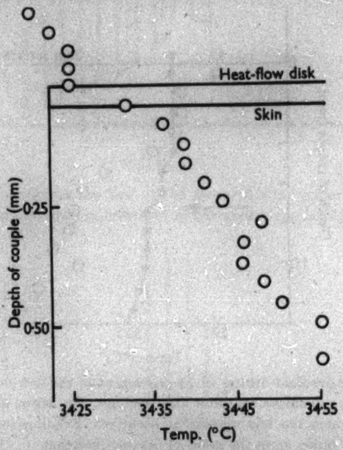

Fig. 15. A skin thermal gradient in a hair follicle. The two parallel lines at the 'skin surface' represent the limits of confidence of depth measurement.

The local skin anatomy was difficult to assess. The figures given by Spalteholtz (1927) were not directly applicable, as they refer to palm, sole and buttock skin and not to front of thigh. Epidermal thickness at the site of the experiments was estimated to be 0·12 mm by measuring the excursion of a microscope focused first on the skin surface, marked with ink and cleared with liquid paraffin, and then on the summits of the capillary loops. The readings varied from 0·10 to 0·15 mm with a mean of 0·12 mm. To assess the relative depths of skin structures skin was taken from the front of thigh of an adult male cadaver of similar build to the experimental subject. Paraffin sections were made after fixation in formol saline. The depth of capillary loops below the surface was from 0·045 to 0·090 mm, indicating shrinkage of the section to one half or one third of the thickness in life. The horny layer of the epidermis was 0·015–0·036 mm deep, the stratum lucidum 0·015 mm and the prickle cell layer 0·018 mm deep over the dermal papillae and 0·060 mm between the papillae which were neither deep nor numerous. Minute vessels occurred within 0·009 mm of the base of the epidermis, but larger vessels with muscular coats first appeared at 0·12–0·15 mm below the surface. Hair follicles penetrated to 0·8 mm at an angle of about 45° and had broad conical mouths filled with horny material.

In the experiment shown the couple was 0·025 mm in diameter, giving a galvanometer deflexion of 4·3 divisions/° C. The heat-flow disk reading was 0·0056 cal/cm^2.sec (200 kcal/sq.in. hr). The room temperature was at 26·44° C and the subject's skin temperature locally was 34·2° C. Calculated thermal conductivities of skin were 0·00086 cal/cm. ° C.sec for the outer 0·3 mm of skin thickness and 0·00043 for the slope of the outermost 0·05 mm of the gradient. These figures are higher than for the punctured gradient. Possible reasons for this, making these results of doubtful significance, are the peculiar anatomy of the follicle, the fact that the subject was sweating sensibly a little and that a larger heat flow than usual was induced to magnify the gradient.

Direct skin puncture results

The skin was punctured between the halves of the box with a glass fibre 0·04 mm in diameter. A little ink on the fibre stained the puncture to assist insertion of the thermocouple. It was found that couples smaller than 0·025 mm would only enter the outer epidermis. Repeated attempts to push them further failed. Couples 0·030 mm in diameter would penetrate epidermis. However, they would not pass further. Couples stiffened with glass fibres to within their last 2 mm would not go further but actually depressed the skin. The glass fibre used in the initial puncture met with as much resistance on its second passage as at first. It seemed that a layer in the dermis rapidly closed after a puncture had been made. One couple only, from the large number used, did probably penetrate the dermis. Others, though not penetrating so deeply, gave closely similar results. Fig. 16 shows the gradient observed. The couple diameter was between 0·035 and 0·040 mm. The subject was seated with the right leg unclothed, wearing two layers of light cotton clothing over the body and arms. After the disturbance caused by cleaning the thigh with alcohol there was a waiting period of 1 hr to allow recovery of equilibrium. The room temperature rose slowly from 22·22 to 22·45° C during this period, falling again to its first value. During the experiment it rose from 22·22 to 22·26° C. The water-bath supplying the apparatus was at 31·57° C. The skin temperature beneath the box was about 32·2° C. The heat-flow disk readings before and after the skin gradient readings were 39·8 and 39·9, corresponding to a heat flow of 0·00204 cal/cm^2.sec (73 kcal/sq.in. hr). No local inflammation was noted with the first puncture. Later attempts to reintroduce the couple caused visible inflammation. The skin temperature then rose at the puncture by 1·5° C.

This gradient gives insufficient evidence on which to base any general conclusion, representing as it does one site on one occasion. However, it does show the form of a skin temperature gradient, with suggestive evidence of a rise in temperature in the dermis (Bazett, 1949). Even allowing for a shrinkage of the control section of skin to one third of the *in vivo* thickness the measurements suggest that this thermocouple reached the subpapillary plexus of muscular vessels. Though a small rise in temperature is recorded in the dermis it is much smaller than the changes described by Bazett (1927, 1941). This suggests that the gradients described by Bazett, though representing the various vascular networks, may be produced partly in response to trauma, and that normal temperature variations with depth in skin are not so marked. The depths of structures involved in skin punctures are difficult to assess without further exact work on the anatomy of the sites of puncture.

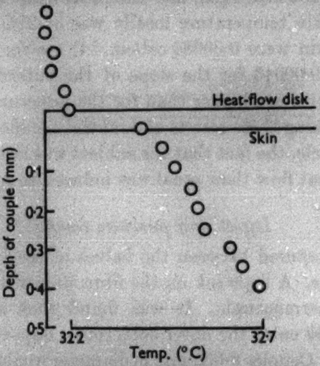

Fig. 16. A skin thermal gradient by direct puncture. The two parallel lines at the 'skin surface' represent the limits of confidence of depth measurement.

This gradient represents a value of 0·00032 cal/cm. °C. sec for the thermal conductivity estimated from the line giving the mean slope in the outermost 0·3 mm of skin (Fig. 16). Slopes for the tissue between 0·2 and 0·3 mm deep and for the outermost 0·05 mm give conductivities of 0·00058 and 0·00019 cal/cm. °C. sec respectively. These figures compare interestingly with those discussed for whole skin and epidermis by Burton & Edholm (1955), being rather lower for epidermis. They suggest that the conductivity falls markedly as the surface is approached, and that the over-all conductivity for skin or epidermis depends on how deep the measurements are taken. The accuracy of the whole estimate is limited to about ± 20% by the depth measurements. Thus the obvious step in temperature which occurs at the heat-flow disk–skin interface in each case may be due either to a surface film of air or to a layer of very low conductivity in the keratinized layer itself or both. In any case the low conductivity of the outermost epidermis suggests that air is included in it. This step in the results at the skin surface emphasizes that it is here that error is maximal both in depth and temperature measurement. In both Figs. 15 and 16 there is a fall at this point of the order of 0·1° C. The results of Grayson (1952) on water content of tissue seem not to be applicable to epidermis, the conductivities found being too low to coincide with the scale for internal organs. The results may represent either a falling water content or increasing air content as the surface is approached. The water content of human epidermis is stated to be about 65% over-all, but to fall variably in the horny layer (Zheutlin & Fox, 1950). Many more experiments would be needed before firm generalizations could be made on these points. These results are presented as preliminary to further work.

SUMMARY

1. A method is described of measuring temperature gradients and heat flows in living skin with minimal local disturbance. Its advantages and disadvantages as compared with other methods are discussed. A very small thermocouple is used.

2. A number of preliminary results is presented. They show the great importance to temperature measurement of small pressures made by apparatus on the skin.

3. Temperature gradients in hair follicles differ from those obtained by direct skin puncture, probably because of differences in local anatomy.

4. Direct-puncture gradients are extremely difficult to obtain. One result is discussed.

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THE EFFECT OF INTRALUMINAL APPLICATION OF 5-HYDROXYTRYPTAMINE AND 5-HYDROXYTRYPTOPHAN ON PERISTALSIS; THE LOCAL PRODUCTION OF 5-HT AND ITS RELEASE IN RELATION TO INTRALUMINAL PRESSURE AND PROPULSIVE ACTIVITY

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In a recent investigation a combined anatomical and physiological study was made of the peristaltic reflex (Bülbring, Lin & Schofield, 1957). Histologically (Schofield, 1957) two distinct types of enteric neurones could be distinguished: (1) unipolar or bipolar neurones, presumably sensory, the processes of which were distributed to the mucosa on the one hand, and to motor neurones on the other; (2) multipolar neurones, presumably motor, the processes of which could be traced to the muscle coats. The submucous plexus of Meissner appeared to be mainly sensory, while Auerbach's plexus appeared to be mainly motor. There was thus morphological evidence for an intrinsic neural pathway between the mucosa and the muscle coats, involving at least one synaptic interruption. The physiological evidence showed that degenerative cutting of all extrinsic nerves had no effect on the peristaltic reflex. However, any procedures interfering with the sensory mucosal fibres, such as removal of the mucous membrane, or asphyxiation, or local anaesthesia of the mucous membrane, abolished the peristaltic reflex.

The anatomical and physiological observations were therefore in agreement and were consistent with the view that the nervous pathway of the peristaltic reflex is entirely intrinsic, and that the mechanism by which a rise of intraluminal pressure triggers the reflex is by stimulation of pressure receptors situated in the mucosa.

It is well known that the intestine is rich in 5-hydroxytryptamine (5-HT) (for references see Erspamer, 1954) and that this is mainly present in the mucous membrane (Feldberg & Toh, 1953). In the whole animal 5-HT causes increased intestinal activity, and the outstanding clinical symptom in patients suffering from carcinoid tumours derived from the argentaffin cells of the mucosa, which contain large amounts of 5-HT (Lembeck, 1953, 1954), is diarrhoea (Lembeck, 1957). 5-HT is a well-known smooth muscle stimulant, but both Kosterlitz & Robinson (1957) and Ginzel (1957), who studied the peristaltic reflex in isolated loops of intestine, found that addition of 5-HT to the bath inhibited or abolished the reflex.

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Considering the finding mentioned above, that the sensory receptors initiating the reflex are situated in the mucosa, it seemed of interest to study the action of 5-HT when applied, not to the serosal but to the mucosal surface. For this purpose a method was required for recording peristalsis in which substances could be introduced into the lumen of an isolated loop of intestine.

The simplest and most widely used method for studying peristalsis in isolated loops of intestine is that of Trendelenburg (1917). It was the first quantitative method, devised to show that the reflex occurred at the moment when the intraluminal pressure reached a certain threshold height. However, the peristaltic reflex easily becomes fatigued and fails if the intraluminal pressure is kept high for some time. For this reason Feldberg & Lin (1949) modified the procedure by raising the pressure for limited periods only, i.e. for one or two minutes at regular intervals of ten to fifteen minutes. Nevertheless, it is often difficult to distinguish unequivocally, from the records obtained, between true peristalsis and muscle contractions of the pendular type. This may lead to a misinterpretation of the results, though it is easy to distinguish between the two types of activity by direct inspection of the piece of intestine itself. In Trendelenburg's method the fluid inside the intestinal lumen cannot be changed and drugs therefore cannot be administered to the mucosal surface without interrupting the record. Baur (1923) and Straub & Triendl (1934), studying the action of purgatives, modified Trendelenburg's method and measured the transport of fluid by a loop of intestine *in situ*. Similar methods were used by Gregory (1950) and by Streeten & Vaughan Williams (1951). Taking advantage of the experience gained by previous workers we arrived at a method which enabled us to test the peristaltic reflex elicited by a rise in intraluminal pressure recurring automatically at a constant rate, to record the pressure changes as well as the peristaltic performance, and to apply various substances to the inside as well as to the outside of the isolated loop of intestine.

A preliminary account of some of the results to be described in this paper was given in a communication to the Physiological Society (Bülbring & Lin, 1957).

METHODS

The apparatus is illustrated in Fig. 1. An isolated loop of intestine was immersed in a bath of approx. 70 ml. capacity, 10 cm long and 2·5 cm high with sloping side walls to reduce the volume, i.e. narrow at the bottom (1 × 10 cm) and wide at the top (4 × 10 cm). The oral end of the intestine was tied to the inflow tube which entered the bath through the wall nearest the pressure bottles; the anal end was tied to the outflow tube which left the bath through the opposite wall. Fluid entered the loop of intestine from two Marriotte bottles (one for normal solution, the other for a test solution) which were connected with a three-way stop-cock to a U-shaped glass tubing which passed through the outer warming bath kept at 37° C. The rising part of the U-shaped inflow tube was a 10 cm length of capillary tubing (inner diameter 1·0 mm). This capillary reduced the rate of inflow. The purpose was threefold: (1) A slow rise of distending pressure gave the intestine time to recover from its preceding activity; early fatigue was thus avoided. (2) A constant rate of rise in intraluminal pressure was important for the determination of threshold pressure. Trendelenburg (1917) showed that the threshold depended largely on the rate at which the pressure was raised.

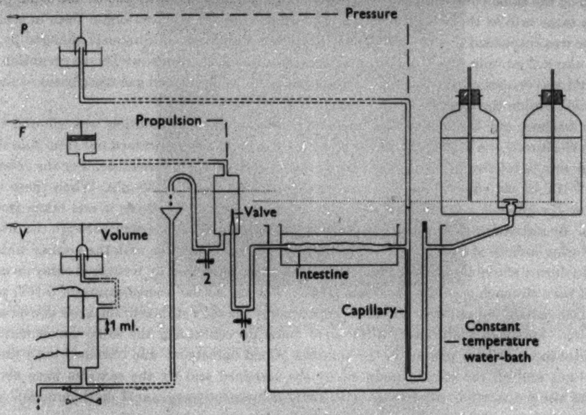

Fig. 1. Diagram of apparatus; for description see text.

(3) With a slow constant inflow it was no longer necessary to raise and lower the pressure intermittently by hand. A water manometer on the inflow side, connected to a float recorder (*P*), recorded the threshold height of intraluminal pressure at which the reflex was initiated and the pressure changes at the oral end produced by the muscle contractions. The float recorder had to be very sensitive to detect differences in threshold of only a few mm water pressure. As a consequence, when a high back pressure developed during the peristaltic contractions, the recorder had to be stopped when it reached the top of the kymograph, as may be noticed in several of the records. Whether the muscle contractions were effective propulsive movements, in contrast for example to spasms or pendular activity, was shown by the records from the outflow side.

The fluid was expelled from the intestine through a rubber valve into a small vertical glass container in which the fluid level was held constant by an overflow device set at a few mm above the inflow pressure. The purpose was threefold: (1) The valve prevented the expelled contents from falling back into the relaxing loop of intestine, which was an advantage compared with Trendelenburg's arrangement in which the regurgitation of fluid was an additional cause of fatigue. (2) Owing to the valve and to the higher outflow pressure no fluid could pass through the intestinal loop unless there was peristaltic activity. (3) The distinction between propulsive muscle contractions and other types of contractions was thus possible. The piston recorder (*F*) connected to the glass container showed whether fluid entered it, i.e. whether a contraction recorded on the inflow side actually led to a transport of fluid. However, as the fluid was siphoned out at the same time as it was coming in, the rise of the piston gave no true record of the volume but rather of the speed at which the fluid was expelled, i.e. the propulsive force. It was collected from the overflow into a relay-operated volume recorder (V) similar to that used by Streeten & Vaughan Williams (1951), which was connected to a float recorder.

For most experiments isolated loops of guinea-pig ileum were used; a few experiments were done on isolated loops of rabbit jejunum. The preparations were obtained immediately after killing the animal by a blow on the head and bleeding it out. For guinea-pig ileum Tyrode solution equilibrated with 95% O_2 + 5% CO_2, for rabbit jejunum Locke's solution oxygenated with pure oxygen, or a modified Krebs's solution (see Bülbring, 1953), were used.

The solution passing through the lumen of the intestine was oxygenated before it was put into the Marriotte bottle. Any substance to be tested was put in the second Marriotte bottle, dissolved in solution of the same composition as the control and reaching the intestine at the same pressure and the same rate as the control solution. The solution in the isolated organ bath in which the intestine was suspended was also previously oxygenated and was continuously changed by a slow flow of about 2 ml./min, the level being kept constant with an overflow. The oxygenation of the bath fluid was continued throughout the experiment by using a small gas-distribution-tube lying in the bath beside the intestine.

After washing out the lumen, the intestine was mounted and solution was allowed to flow through the lumen at a low subthreshold pressure. The solution was drained out from side tube (1) for 20–30 min to remove mucus, before raising the pressure to elicit peristalsis. For the determination of 5-HT in the effluent, samples were collected for periods of 30 min. When there was no peristalsis the effluent was collected from side tube (1); during peristalsis it was taken from side tube (2). By restricting the outflow from tube (1) the volume of fluid passing through the intestinal lumen during periods of inactivity could be adjusted to be comparable with the volume which was propelled during active peristalsis. The 5-HT content was estimated by biological assay on isolated strips of rat's stomach according to Vane (1957). For the actual identification of 5-HT, parallel assays were carried out on three different preparations, the rat's stomach, the rat's uterus and the rat's colon. Standard solutions of 5-HT were made up containing the same concentrations of other substances as were present in the samples. Good agreement was obtained with the three preparations and all the effects produced by the standard and by the samples were abolished when the test preparation was treated with BOL (2-brom-D-lysergic acid diethylamide).

RESULTS

Effect on peristalsis

Normal peristalsis. Guinea-pig ileum normally exhibited vigorous peristalsis when the pressure was raised to about 20 mm water. The threshold pressure in a fresh preparation was usually very low (between 10 and 15 mm) but rose within the first 30 min to a steady level; few preparations required more than 20 mm. Contractions were often frequent during the first 15 min of the experiment and then gradually became slower. They remained regular in most preparations, but in some experiments groups of 2–15 contractions alternated with pauses. Gradually, over a period of many hours, peristaltic activity declined. The change in the efficiency of peristalsis is shown in Fig. 2. At the beginning of the experiment the threshold pressure was between 16 and 18 mm water and very little fluid was pushed backwards (*a*); therefore the pressure changes during the peristaltic contractions recorded from the oral end were small, while the excursions of the piston record from the outflow container were large, indicating rapid and efficient propulsion of fluid. In (*b*) taken about 1 hr later, the threshold had risen to about 20 mm, the pressure changes at the oral end had become larger, some fluid being pushed backwards, and the records from the outflow side had become correspondingly smaller. Though the number of contractions was no less in (*b*) than in (*a*), the volume of fluid transported was reduced, as shown in the bottom record.

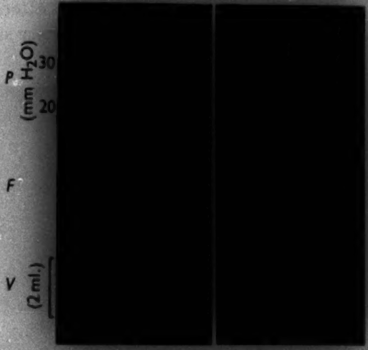

Fig. 2. Records of peristaltic activity of isolated guinea-pig ileum. Top, *P* = pressure (mm water) at oral end; middle, *F* = fluid propulsion; lower, *V* = volume transported (ml.). Time marker, 30 sec. (*a*) at beginning of experiment, (*b*) about 1 hr later.

The effect of 5-*HT on peristalsis*. When 5-HT was added to the fluid passing through the lumen of the intestine it stimulated peristalsis. In concentrations from 10^{-9} to 10^{-5} 5-HT lowered the threshold pressure, it increased the frequency of contractions and it increased the volume of fluid transported. Lower concentrations had no effect, higher concentrations were not tested. Since the rate of inflow was constant throughout each experiment a lowering of threshold allowed the critical intraluminal pressure to be reached sooner; consequently contractions were elicited more frequently. A typical record is shown in Fig. 3. Normal peristaltic contractions occurred at a threshold (marked with dots) of 19–21 mm water, and the fluid transport was about 1 ml./min. When 5×10^{-7} 5-HT reached the lumen the threshold fell to 13 mm and the fluid transport was doubled. The full effect was reached within 4 min but, though 5-HT was still present in the solution passing through the lumen, the preparation adapted and after 10 min peristalsis gradually slowed again.

Concentrations from 10^{-9} to 10^{-5} were also administered to the outside by adding 5-HT to the bath fluid. In no concentration was peristalsis stimulated but it was always inhibited. While lower concentrations caused only temporary inhibition higher concentrations abolished the reflex. In some preparations, however, peristalsis returned spontaneously after 20–30 min while the 5-HT was still present in the bath. Nevertheless, when administered outside the effect of 5-HT was invariably inhibitory, whereas when administered inside its effect was stimulant.

In the experiment shown in Fig. 4, 10^{-6} 5-HT was applied to the mucosa, (*a*), for only 8 min. Its effect on threshold and peristaltic activity occurred immediately but was equally quickly abolished as soon as solution containing no 5-HT reached the lumen. Adaptation had already begun, but after washing out the 5-HT peristalsis stopped for several minutes. When activity started again, the contractions were inefficient, producing high back pressure and no fluid propulsion. Ten minutes later 5-HT, in only half the concentration of that before, was applied to the serosa (*b*). It caused temporary cessation of peristalsis which was, however, resumed spontaneously in the presence of 5-HT, the fluid transport being again inefficient. Thus both the removal of 5-HT from the inside, and the addition of 5-HT outside inhibited peristalsis. These pauses were, however, different from periods of inactivity alternating with periods of peristalsis, which occurred in some preparations spontaneously (as in Fig. 12), and also during prolonged perfusion of the lumen with high concentrations of 5-HT, as in Fig. 5. This shows records taken from a preparation which responded to an intraluminal pressure of 20 mm, after a latent period of 1–2 min, with two contractions each expelling about 1 ml. fluid. When 10^{-6} 5-HT reached the lumen, (*a*), the lowering of threshold led to an outburst of activity. The pattern of peristalsis was changed; while 5-HT was present, peristaltic bursts alternated with equally long intervals, (*b*). They recurred undiminished until the 5-HT was washed out, and the initial pattern of peristalsis was resumed, (*c*). When now the same concentration of 5-HT as before was added to the bath, peristalsis ceased immediately, but slowly returned on removal of the 5-HT from the bath. At this time, (*d*), the stimulant action from inside was readily repeated.

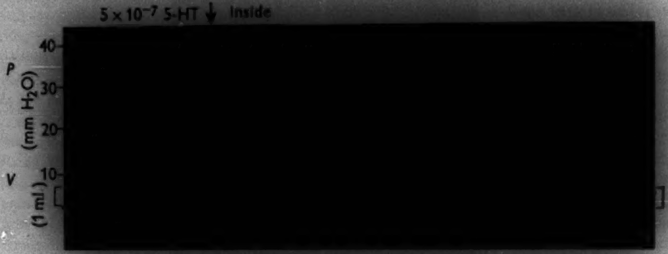

Fig. 3. The effect of 5-HT, 5×10^{-7}, inside. Records *P* and *V* as in Fig. 2. Threshold of intraluminal pressure at which contractions occurred marked with dots. Note lowering of threshold, increased fluid transport; and adaptation. Time marker, 30 sec.

During the time in which 5-HT exerted its inhibitory action from outside, it was impossible to stimulate peristalsis with 5-HT from inside. Similarly, during the time in which 5-HT produced its stimulant action from inside, this could immediately be interrupted by applying 5-HT outside. When peristalsis was abolished by removing the mucosa from a loop of intestine or by introducing cocaine or procaine into the lumen, the administration of 5-HT inside produced no effect.

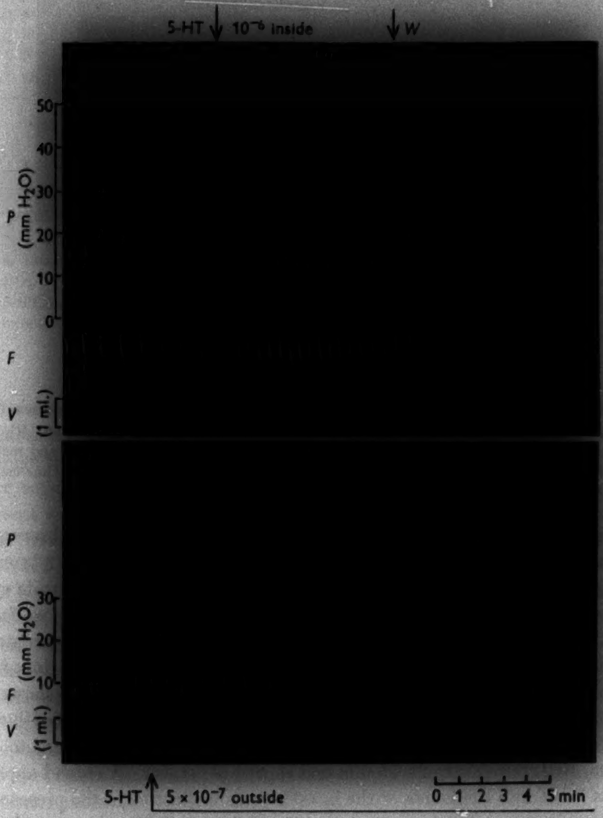

Fig. 4. Stimulating effect of short application of 5-HT 10^{-6} inside (*a*) and inhibitory effect when 5-HT was washed out from inside. Note that weaker concentration of 5-HT outside (*b*) caused transient inhibition. Records as in Fig. 2.

The effect of 5-HT 10^{-7}–10^{-5} on rabbit jejunum was studied in only four experiments. The stimulating effect of 5-HT introduced into the lumen was less readily obtainable than in guinea-pig ileum. It developed more slowly, was less marked, more transient and, though the contractions became more frequent, the volume of fluid expelled was not always increased. When 5-HT was applied outside it did not stop peristalsis in rabbit jejunum.

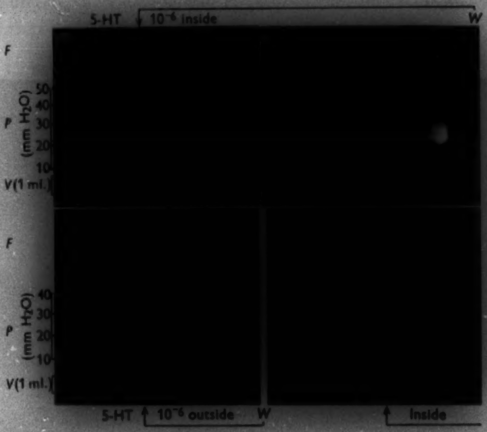

Fig. 5. Prolonged application of 5-HT 10^{-6} inside, (*a* and *b*), produced bursts of peristaltic activity alternating with pauses. Initial pattern of peristalsis returned after washing out, (*c*), until abolished by 5-HT 10^{-6} outside for 20 min. Recovery, (*d*), after washing out and renewed stimulation by 5-HT inside. Records as in Fig. 2 but in the order of *F*, *P*, *V*. Time marker, 30 sec.

The effect of antagonists of 5-HT on peristalsis. We confirmed the observation of Ginzel (1957) that LSD (lysergic acid diethylamide) and BOL (2-brom-D-lysergic acid diethylamide) 10^{-6}–10^{-5}, when administered to the outside of an isolated loop of intestine, abolished the peristaltic reflex. However, we found that the action of lower concentrations was only temporary and that peristaltic activity, though reduced, returned after some 10–30 min. Concentrations from 10^{-7} to 10^{-5} of LSD and BOL were also administered inside. Fig. 6 shows the effect of 5-HT 10^{-6} compared with that of BOL 10^{-5}, both applied inside. While 5-HT lowered the threshold from 20–22 mm water pressure to 15–16 mm, BOL raised it to 23–26 mm and slowed peristalsis. A second application of 5-HT into the lumen of the intestine was ineffective in the presence of BOL; peristaltic activity continued unchanged until BOL was now administered to the outside; this arrested the activity immediately. After removal of BOL from the solution in the bath, peristalsis returned, (*W*), though BOL 10^{-5}

was still present inside the lumen. Thus we found that the action of both antagonists was the same as that of 5-HT when administered outside. On the other hand, applied inside, both antagonists exerted the opposite effect to that of 5-HT, raising the threshold, slowing the rate of contractions and reducing the volume of fluid transported. This effect suggested that their action was due to an antagonism of 5-HT which might be released locally.

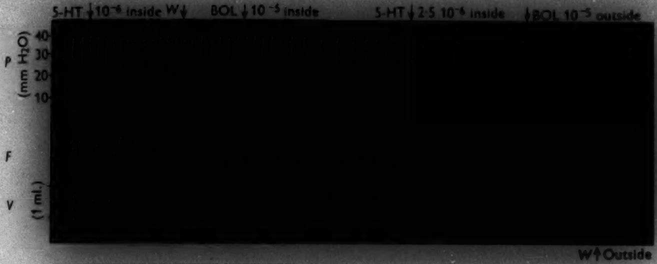

Fig. 6. Lowering of threshold (marked with dots) by 5-HT 10^{-6} inside, and raising of threshold by BOL 10^{-5} inside; this also abolished the action of 5-HT. Note increased fluid transport with 5-HT, and diminished fluid transport with BOL. Abolition of peristalsis only with BOL 10^{-5} outside. Records as in Fig. 2. Time marker, 30 sec.

The release of 5-*HT*

The release of 5-*HT into the fluid passing through the lumen of the intestine.* Small amounts of 5-HT were found to appear in the effluent after its passage through the lumen of the small intestine of guinea-pigs and of rabbits. Samples were collected for periods of 30 min, the volume was measured, the 5-HT content was determined on the rat's stomach preparation and the release of 5-HT/30 min was calculated. The results are summarized in Table 1. Though the 5-HT release varied from one experiment to another it was found that in each individual experiment there was a correlation between the intraluminal pressure, the release of 5-HT and the volume of fluid transported. The individual variation was such that in some preparations the 5-HT release at subthreshold pressure was as high as that in others at an intraluminal pressure of 30 mm with active peristalsis. The resting 5-HT release ranged from 20 to 420 ng in 30 min; during peristalsis it ranged from 107 to 1100 ng in 30 min. In spite of this overlap the single experiments as well as the mean figures indicated that a rise in intraluminal pressure to 17–20 mm, which was just above the threshold level, increased the 5-HT release to two to three times the resting value, while a further rise to 25–30 mm increased the 5-HT release to three to four times the resting value. The amount of fluid transported rose in the same proportion. However, it appeared that the 5-HT release was independent of the rate at which the fluid was washed through the lumen. In the experiment shown in Fig. 7 the volume either actively transported or passively passed through the lumen was very similar during seven of the nine 30 min periods, i.e. 39 ml. ± 4 ml., yet the 5-HT content was very different. In the experiment shown in Fig. 8 the volume of fluid transported during the first period of active peristalsis was actually smaller than that flowing through the lumen during the two rest periods before and after, but the 5-HT content was much greater.

TABLE 1. Release of 5-HT and transport of fluid by isolated guinea-pig ileum (Expts. 1–17) and rabbit jejunum (Expts. 18–20) during 30 min

	Subthreshold pressure		17–20 mm		25–30 mm	
Expt.	HT released (ng)	Fluid passed through lumen (ml.)	HT released (ng)	Fluid expelled (ml.)	HT released (ng)	Fluid expelled (ml.)
1	300	46	660	66	—	—
2	225	40	362	52	—	—
3	135	45	434	79	—	—
4	420	30	600	30	—	—
5	—	—	712	62	—	—
6	—	—	210	33	337	42
7	207	37	445	46	637	49
8	192	50	385	31	472	56
9	310	52	—	—	975	195
10	160	52	—	—	685	137
11	170	46	—	—	200	80
12	50	75	162	75	—	—
13	20	67	—	—	1100	147
14	24	40	107	97	—	—
15	24	48	240	80	—	—
16	60	40	193	100	—	—
17	140	57	388	88	—	—
18	134	52	—	—	449	113
19	174	48	—	—	540	138
20	68	57	—	—	840	112
Mean	156	50	377	65	625	107

Moreover, in the same experiment, the volume collected during the first rest period was as large as that collected during the last active period at high pressure, but during the latter twice the amount of 5-HT was released. The large variation in the absolute amounts of 5-HT found in the effluent of different preparations might be attributed to differences in its formation, storage or destruction. For example, in the experiment illustrated in Fig. 7 two samples were collected during the first hour at an intraluminal pressure of 20 mm when there was active peristalsis. They contained more 5-HT than the subsequent samples collected at subthreshold pressure when there was no peristalsis. Two samples collected during the third hour at an intraluminal pressure of 17 mm contained more than those at low pressure, but also more than the first samples at high pressure. It is possible that some synthesis and storage of 5-HT had occurred during the rest period and that this was then released when the pressure was raised. It may be noted, however, that the initially greatly increased amount of 5-HT rapidly diminished. When the pressure was raised to 30 mm a further increase in 5-HT release occurred and this also declined during the next 30 min. In most preparations a steady decrease with time of the amount of 5-HT released, even during rest periods, was observed. This is shown by the experiment illustrated in Fig. 8 in which the release of 5-HT during the second of two rest periods was much less than during the first, though it could still be greatly increased by raising the intraluminal pressure.

When the pressure was kept high over longer periods the decline of 5-HT release was very rapid, as shown in Table 2. Though the amounts of 5-HT released during the first half hour were very different in different preparations,

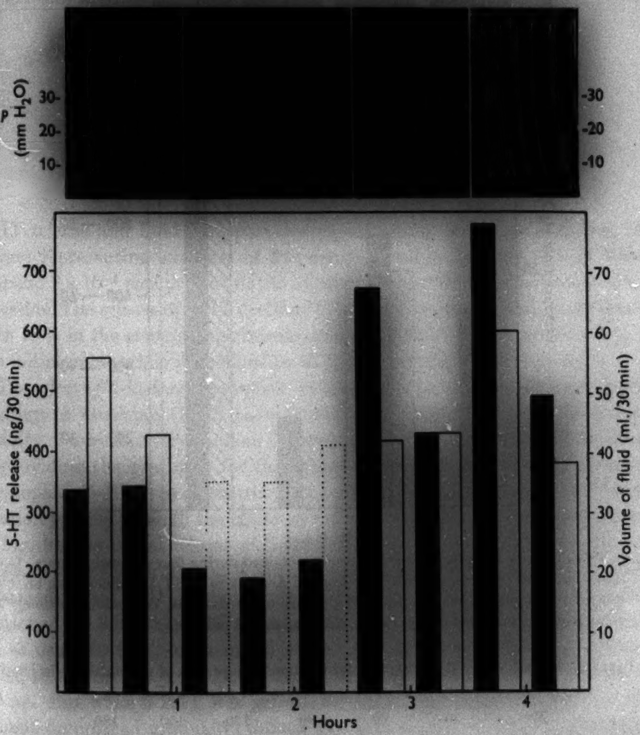

Fig. 7. Tracings show pressure record during peristalsis (*a*) at 20 mm, (*b*) at 7 mm which was sub-threshold, (*c*) at 17 mm, (*d*) at 30 mm. Graph shows, during these same periods, release of 5-HT (ng/30 min, black columns); volume of fluid expelled (ml./30 min, white columns, continuous outline); and volume of fluid collected at rest (white columns, dotted outline).

those with a high initial release showed a much steeper decline and during the next half hour the amounts released were similar in all five experiments. After 1½ hr peristalsis the amount of 5-HT was less than half and after 2½ hr it was reduced to only 10% of the initial value. The volume of fluid transported in these experiments fell much less rapidly and, though after 3 hr the amount of 5-HT appearing in the effluent had fallen to 10% of its initial value, the fluid transport remained steady at about 65% of its initial level (see Table 3).

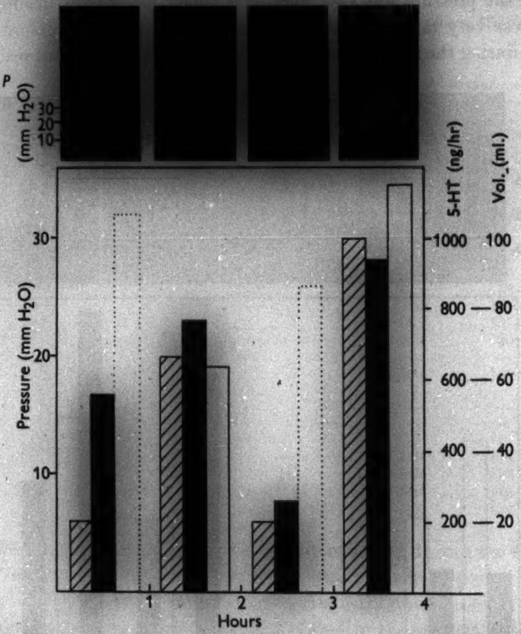

Fig. 8. Tracings show samples of pressure records obtained during four successive hours. Graph shows pressure (mm water, shaded columns), amount of 5-HT in effluent (ng/hr, black columns); volume of fluid transported (ml./hr, white columns, continuous outline); and volume of fluid collected at rest (white columns, dotted outline).

TABLE 2. Release of 5-HT (ng/30 min) during successive periods of peristalsis by isolated guinea-pig ileum (Expts. 1–4) and rabbit jejunum (Expt. 5)

Min...	30	60	90	120	150	180
Expt. 1	1444	225	135	—	—	—
2	554	360	240	—	—	—
3	311	170	106	74	35	27
4	525	260	110	96	—	—
5	840	220	325	200	64	—

The effect of Marsilid on the release of 5-*HT and on peristalsis.* In order to test the effect of preventing the destruction of 5-HT we used Marsilid (Roche Products, 1-*iso*nicotinyl-2-*iso*propylhydrazine) to inhibit amine oxidase. In all experiments it was added to the fluid passing through the lumen of the intestine in a concentration of 2×10^{-4}. It increased the amount of 5-HT appearing in the effluent in only one out of six experiments, but it slowed the otherwise rapid decline of 5-HT release. At the same time peristaltic activity increased gradually over a period of several hours. In Table 3 the percentage change in 5-HT release and fluid transport during continued peristalsis is shown, the figures representing averages of five controls and six experiments in which Marsilid 2×10^{-4} was present in the solution passing through the lumen of the intestine. The release of 5-HT declined to 30 % of the initial value as compared with 10 % in the control experiments, and the fluid transport rose to 168 % of the initial value while it declined to 65 % in the normal preparations.

TABLE 3. The effect of Marsilid on the amount of 5-HT released and on the volume of fluid transported by isolated loops of intestine during successive periods of peristalsis. Figures are expressed as percentages of the first sample

	Controls		Marsilid	
Time (min)	5-HT released	Fluid transport	5-HT released	Fluid transport
30	100	100	100	100
60	42	82	63	102
90	29	62	57	108
120	22	63	42	108
150	10	65	25	135
180	9	65	30	168

The effect of 5-*hydroxytryptophan on the release of* 5-*HT and on peristalsis.* Gaddum & Giarman (1956) have shown that intestinal extracts show high activity of 5-hydroxytryptophan decarboxylase. We therefore tested the effect of providing the precursor for the synthesis of 5-HT by adding 5-hydroxytryptophan (5-HTP) in concentrations ranging from 2×10^{-6} to 2×10^{-5} to the solution passing through the lumen of the intestine. In the experiment shown in Fig. 9*a* the initial release of 5-HT during inactivity at low intraluminal pressure was 24 ng in 30 min. When the pressure was raised to 20 mm the 5-HT release increased to 311 ng, but, while the pressure was kept high and peristalsis continued for 3 hr, the 5-HT release declined to 27 ng and the fluid transport fell as usual to 65 % of its initial value. At this point 5-HTP 2×10^{-6} was added to the perfusion fluid and caused an immediate rise in 5-HT release to 140 ng. But while 5-HTP was present for 2·5 hr, the amount of 5-HT in the effluent diminished gradually, though the fluid transport was maintained at 82 % of its initial volume. In another experiment, shown in Fig. 9*b*, a ten times greater concentration of 5-HTP was added to the perfusion fluid (note the different scale of the ordinate). The spontaneous release of 5-HT in this experiment was the same as in (*a*). By raising the intraluminal pressure to 20 mm the resting value of 24 ng was increased to 126 ng in the first 30 min, but it fell to 88 ng during the second 30 min. In the presence of 5-HTP 2×10^{-5} the amount of 5-HT appearing in the effluent was increased more than 100 times, i.e. to 9750 ng in the first 30 min. This release declined after 5·5 hr to 175 ng, an amount scarcely greater than that released in the beginning of the experiment in response to the rise in pressure. After removal of the 5-HTP in the last hour the release fell to 50 ng. It was surprising to find that, in spite of the continued presence of 5-HTP in high concentration, the release of 5-HT did not remain at a high level. Nor was the fluid transport increased above normal, the greatest volume being 68% of the initial value. This could be due either to rapid destruction of the large quantities of 5-HT by amine oxidase, or to the lack of other essential factors for 5-HT formation.

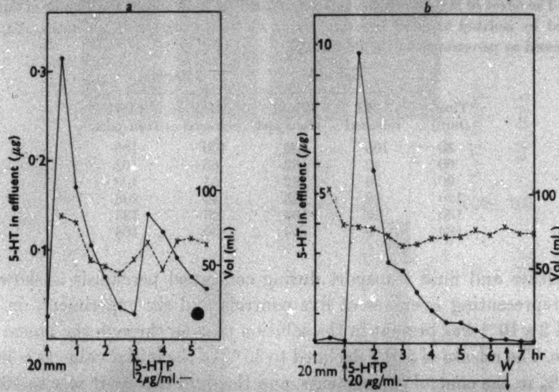

Fig. 9. The effect of adding 5-HTP to the fluid passing through the intestinal lumen on the amount of 5-HT in the effluent (●—●), and on the fluid transport (× - - - ×). Note different scale for release of 5-HT which, though similar in both experiments in response to the rise in intraluminal pressure (20 mm), was raised 30 times more in (*b*) than in (*a*).

The effect of 5-hydroxytryptophan in the presence of Marsilid. As shown in the previous paragraph, the effect of 5-HTP on release of 5-HT was striking, though not maintained, but on peristaltic activity there was little effect. If, however, Marsilid was also present, 5-HTP increased peristaltic activity immediately, as illustrated in the records of Fig. 10, taken from Expt. 2 in Table 4. In (*a*) peristalsis is shown in the presence of Marsilid 2×10^{-4}. When 5-HTP 2×10^{-5} was added to the solution passing through the lumen and containing Marsilid, (*b*), a prolonged stimulation of peristalsis was observed as shown in Fig. 10*b*. Nevertheless, even in the presence of Marsilid, the effect of 5-HTP was transient.

In spite of the large amounts of 5-HT released the volume of fluid expelled by the intestine soon diminished as contractions gradually became less frequent. The mean results of all these experiments are presented graphically in Fig. 11. It shows that the presence of Marsilid inside the lumen delayed the gradual fall in release of 5-HT and increased the propulsive activity of the intestine. It also shows that the additional presence of 5-HTP caused a large increase in the amount of 5-HT released but, at the same time, a diminution of the propulsive activity and the volume of fluid transported. When both Marsilid and 5-HTP were washed out, the 5-HT content in the effluent rapidly declined, but the

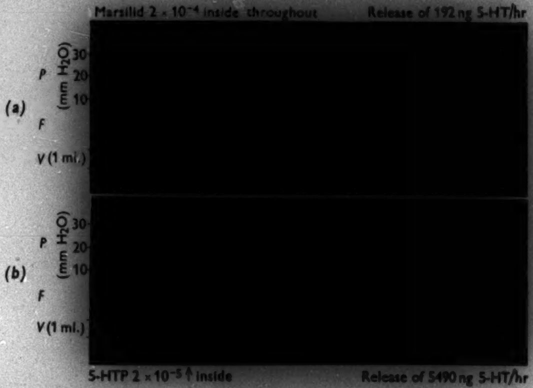

Fig. 10. The effect of 5-HTP inside, in the presence of Marsilid. Records as in Fig. 2. Time marker, 30 sec.

TABLE 4. The effect of Marsilid (2×10^{-4}) and 5-HTP (2×10^{-5}) on the release of 5-HT (ng/30 min) from isolated guinea-pig ileum (Expts. 1–5, 10) and rabbit jejunum (Expts. 6–9)

xpt....	1	2	3	4	5	6	7	8	9	10	
ressure...	High	High	High	High	High	High	High	High	High	Alternately low and high	
rmal solution	—	—	1,444	—	—	—	—	—	—	—	
	358	468	225	410	177	570	316	1170	2250	50	337
	367	400	135	300	118	176	210	920	470	—	100
rsilid	680	120	│	290	│	│	│	450	206	37	105
	386	72	│	│	│	│	│	487	235	48	105
	│	│	│	│	│	│	│	320	230	38	110
TP	↓	↓	648	↓	↓	↓	↓	↓	—	│	│
th	6,750	3250	6,900	9000	12,000	790	775	630	—	│	│
	11,250	2240	16,000	—	11,000	│	1220	938	—	│	│
	↓	1400	—	—	↓	↓	↓	↓	—	↓	↓
shing out	1,800	—	—	—	5,400	780	1435	310	—	30	100
	—	—	—	—	210	260	—	300	—	20	48
	—	—	—	—	214	—	—	252	—	—	—
	—	—	—	—	155	—	—	—	—	—	—

propulsive activity showed a sharp transitory rise indicating that an inhibitory effect was now removed. Fig. 12 also illustrates the stimulation of peristalsis which was followed by inhibition when, in the presence of Marsilid and 5-HTP, excessive amounts of 5-HT were produced inside the lumen of the intestine. Moreover, in other experiments it was found that addition of 5-HT in concentrations which normally caused stimulation had either no effect or inhibited peristalsis when Marsilid and 5-HTP were also present. It may be that Marsilid, preventing the destruction of 5-HT, allowed it to penetrate from the lumen through the mucosa to the outer structures where it exerted a paralytic effect. When 5-HT was given outside in the presence of Marsilid it abolished peristalsis completely, while in the absence of Marsilid the inhibition caused

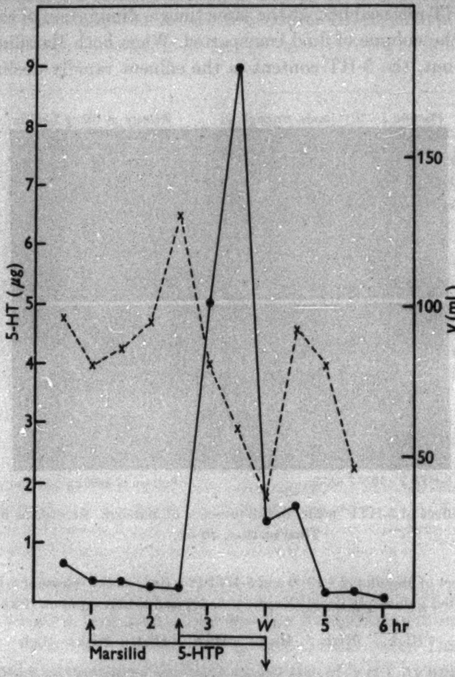

Fig. 11. The effect of introducing Marsilid and, in addition, 5-HTP into the intestinal lumen, on the amount of 5-HT appearing in the effluent (●—●), and on fluid transport (× - - - ×). Note that the production of large amounts of 5-HT in the presence of Marsilid inhibited peristalsis, and that this inhibition was removed by washing out.

by 5-HT, especially in low concentration from 10^{-9} to 10^{-7}, was often only temporary.

The effect of 5-HTP with the addition of pyridoxal. As Marsilid did not prevent the decline of 5-HT appearing in the effluent when the precursor 5-HTP was also present, the failure to maintain a high concentration of 5-HT in the lumen of the intestine was apparently not due to rapid destruction of 5-HT.

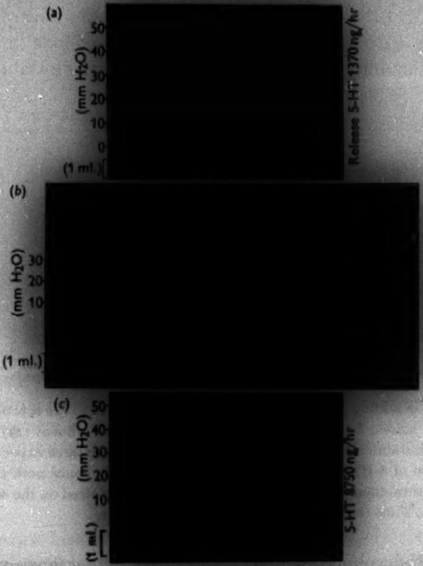

Fig. 12. The effect of simultaneous addition of 5-HTP and Marsilid. Records are continuous: (*a*) before; (*b*) when 5-HTP 2×10^{-5} + Marsilid 2×10^{-4} reached the intestinal lumen; (*c*) 15 min later. Time marker, 30 sec. Note the initial increase followed by a reduction of peristalsis.

We tested, therefore, the possibility that another essential substance in the formation was lacking, by adding not only 5-HTP, but also the material required for the co-enzyme of decarboxylation of 5-HTP, i.e. pyridoxal hydrochloride, in a concentration of 10^{-5}. Three experiments in which three different concentrations of 5-HTP were used are shown in Fig. 13. In each experiment the first sample was collected while there was no peristaltic activity, and then the spontaneous increase in 5-HT release produced by raising the intraluminal pressure was determined. After 60 or 120 min 5-HTP was added and, in (*a*) and (*b*), pyridoxal was added 2·5 hr later, while in (*c*) both substances were added simultaneously. In (*a*), when $1{\cdot}5\times10^{-5}$ 5-HTP was present, pyridoxal prevented the decline of 5-HT release. Indeed, this was maintained at a level well above the initial value recorded after raising the intraluminal pressure. The number of contractions and the volume of fluid transported were also increased. When the lumen was washed out with normal solution there was a steep fall in the amount of 5-HT appearing in the effluent, giving an indication of the extent to which the addition of 5-HTP and pyridoxal had enabled the intestine to synthesize 5-HT and to maintain peristaltic activity. In Fig. 13*b* the initial rise in 5-HT production caused by 5-HTP was greater than in (*a*), but though it rapidly declined pyridoxal prevented it from falling below the initial value produced on raising the intraluminal pressure.

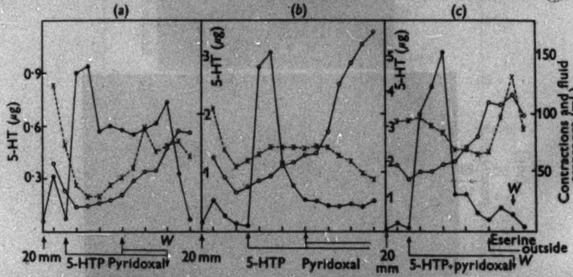

Fig. 13. The effect of adding pyridoxal, in the presence of 5-HTP, to the solution passing through the intestinal lumen. The amount of 5-HT appearing in the effluent (●) in response to the initial rise in intraluminal pressure (20 mm) was similar in the three experiments (*a*, *b*, *c*), but the production of 5-HT was differently affected by 5-HTP, hence note the different scales. Number of contractions (○) and fluid transport (×) were plotted on the same scale throughout. Abscissa: Time (hr). For detailed description see text.

Nevertheless, the volume of fluid transported fell progressively. This was, however, not due to a slower rate of contractions as in the presence of Marsilid + 5-HTP. On the contrary, the number of contractions rose from 2/min to nearly 6/min, yet the propulsive force diminished. Fig. 14 shows records taken from the same experiment as Fig. 13*b* at 1 hr intervals. While the threshold of intraluminal pressure rose during the first 2 hr of peristalsis the addition of 5-HTP lowered the threshold and the additional presence of pyridoxal lowered it further. Consequently peristaltic contractions were elicited at a very high rate, but there was a decline in fluid transport, presumably because of muscular fatigue or failing acetylcholine synthesis. In the experiment of Fig. 13*c* this point was tested. By simultaneously infusing 5-HTP and pyridoxal the 5-HT release was maintained at a level above the initial spontaneous release. But though the contractions became more frequent, the volume of fluid expelled became less. When eserine, in a low concentration, 6×10^{-9}, was added to the outside fluid in the bath, the force of muscle contractions increased immediately and the transport of fluid now became proportional to the rate of muscular activity.

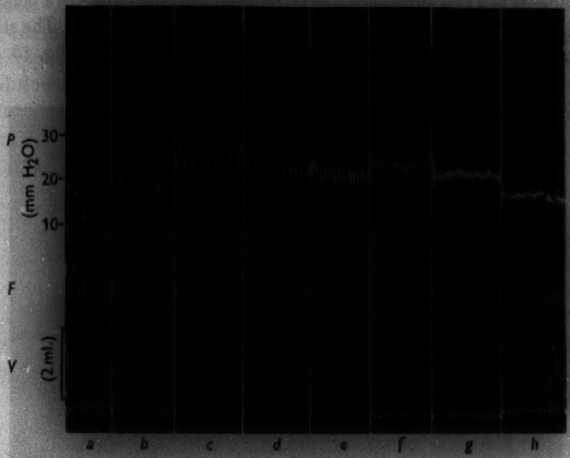

Fig. 14. Records (as in Fig. 2) at hourly intervals of peristalsis before (*a* and *b*), during the presence of 5-HTP inside the intestinal lumen (*c*, *d*, *e*), and during the additional presence of pyridoxal (*f*, *g*, *h*). Time marker, 30 sec. Note the rising threshold in the pressure record (top) before, and the progressively falling threshold after addition of 5-HTP and pyridoxal. Same experiment as Fig. 13*b*.

The effect of other drugs

Acetylcholine and histamine. In order to test whether the action of 5-HT on peristalsis was specific or whether it was similar to that of other substances which stimulate smooth muscle, we applied acetylcholine (ACh) in concentrations from 10^{-7} to 10^{-5} and histamine in concentrations from 10^{-7} to 10^{-6} to the outside as well as to the inside of the intestine. When added to the solution in the bath, both substances stimulated peristalsis in concentrations of 10^{-7}. The threshold was lowered, contractions became more frequent and more fluid was transported. ACh 10^{-5} and histamine 10^{-6} applied outside caused spasm of the muscle. Peristaltic activity was prevented because no fluid could enter the tightly constricted lumen. After washing out there followed a prolonged period of paralysis in which the reflex could no longer be elicited. When contractions returned they remained for a long time irregular and incoordinated. When ACh was introduced into the lumen of the intestine it produced the same effect as from the outside, stimulating peristalsis in low concentrations and inhibiting it in high concentrations. Histamine produced no effect from inside.

The effect of phenyldiguanide. On the assumption that 5-HT exerted its action by stimulating sensory receptors it was of interest to test the action of other substances which stimulate sensory endings, one of them being phenyldiguanide. When this substance was introduced into the lumen in a concentration of 2×10^{-4} it stimulated peristalsis in the same way as 5-HT. The stimulation was followed by a slowing of peristalsis. This is shown in Fig. 15.

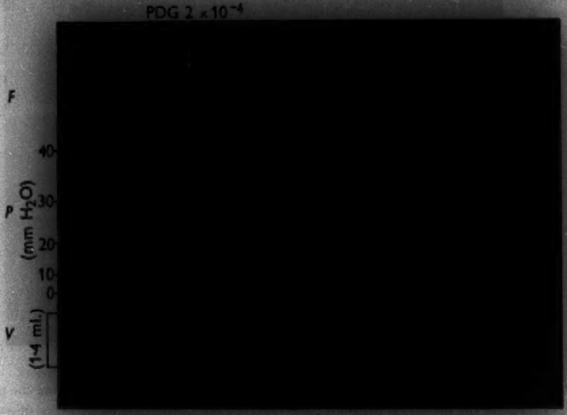

Fig. 15. The effect of phenyldiguanide 2×10^{-4} in the solution passing through the intestinal lumen. Records as in Fig. 2, but in the order *F*, *P*, *V*. Time marker, 30 sec.

In other preparations of guinea-pig ileum, in which the longitudinal muscle contractions were recorded, it was found that phenyldiguanide caused ganglion block. In a concentration of 3×10^{-4} it abolished the action of nicotine, leaving the action of ACh unaffected.

The effect of procaine on the release of 5-*HT*. Feldberg & Lin (1949) showed that local anaesthetics applied outside abolished the peristaltic reflex by their ganglion-blocking action. Recently we found that if cocaine was introduced into the lumen in concentrations which did not affect ganglionic transmission it abolished, nevertheless, the peristaltic reflex, presumably by blocking the sensory receptors (Bülbring, Lin & Schofield, 1957). In order to answer the question whether 5-HT was released primarily as a result of raised intraluminal pressure, or whether the release was a secondary effect produced by peristaltic activity, we investigated the action of procaine. Fig. 16 shows the effect on peristalsis. In the experiment illustrated in (*a*) and (*b*) procaine was administered outside. It abolished peristalsis but it did not abolish muscular activity. Whereas in (*a*), at an intraluminal pressure of 20 mm, propulsive activity was observed, there was in (*b*) no longer any propulsion of fluid, though the intraluminal pressure had risen above 30 mm and the muscle contracted vigorously at high frequency. Similarly, (*c*), when procaine was given inside it stopped peristalsis, but as the pressure rose above 30 mm many muscle contractions were seen, yet no fluid was expelled. In both these conditions the release of 5-HT was measured. After procaine had stopped peristalsis the fluid was collected from side tube (1) (see Fig. 1), and the outflow was adjusted so that the rate of flow of fluid through the lumen was kept constant within 10%. By alternately raising and lowering the pressure, its influence on release of 5-HT

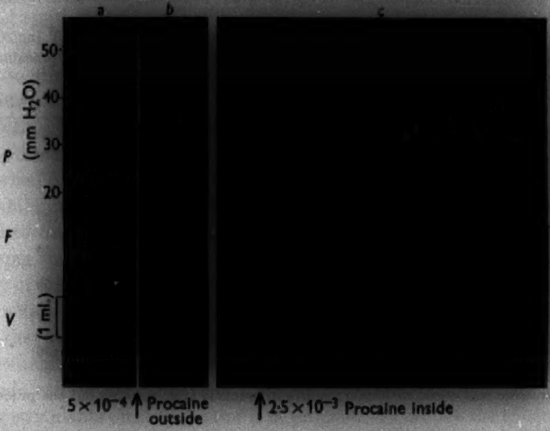

Fig. 16. Records (as in Fig. 2) of peristalsis, (*a*) before, (*b*) in the presence of procaine outside; (no volume record), (*c*) another experiment showing the effect of procaine inside the intestinal lumen. Time marker, 30 sec. Note, in both experiments, muscle contractions which fail to expel fluid.

was determined. The results are given in Table 5. In Expts. 1 and 2 the procaine concentration inside the lumen was $2{\cdot}5\times10^{-3}$, in Expt. 3 it was 5×10^{-4}; and in Expt. 4 procaine 5×10^{-4} was applied outside. In order to follow the course of the experiments it is necessary to find the figures in successive 30 min periods of Table 5. Thus Expt. 1 was started with five periods at high pressure, which was then lowered for two periods and then raised again. The other three experiments were started with a period of low pressure and this alternated every 30 min with periods of high pressure. In each experiment procaine at first reduced the 5-HT release, but in those experiments (1, 2 and 3), in which procaine was present in the solution passing through the lumen, the amount of 5-HT gradually increased again, in one experiment above the initial value obtained by raising the pressure, in the other experiments to 60 and 40%, respectively. When, in Expt. 3, the procaine was washed out from the lumen there was a sudden fall in 5-HT release though at that time peristaltic activity had returned. The observation that in the presence of procaine inside the lumen of the intestine the release of 5-HT was maintained at a high level was reminiscent of the effect of Marsilid and may be attributed to the inhibitory action on amine oxidase. This interpretation is supported by the results of Expt. 4, in which procaine was given outside and in which the release of 5-HT declined to about 10 % of its initial value as it does in normal solution. Procaine reduced the extent to which the 5-HT output changed on increasing the intraluminal pressure; it did not, however, abolish the correlation.

TABLE 5. Effect of raising the intraluminal pressure on the release of 5-HT (ng/30 min) in the presence of procaine inside the lumen (Expts. 1, 2 and 3; i), and of procaine outside (Expt. 4; o)

Time	1 (i)		2 (i)		3 (i)		4 (o)	
(min)	8 mm	25 mm	8 mm	25 mm	8 mm	25 mm	8 mm	25 mm
30	—	576	310	—	160	—	120	—
60	—	544	—	975	—	685	—	1100
				Procaine				
90	—	466	230	—	140	—	127	—
120	—	510	—	360	—	192	—	340
150	—	660	231	—	157	—	60	—
180	440	—	—	414	—	220	—	115
210	475	—	450	—	138	—	59	—
240	—	870*	—	575	—	255	—	65
270	—	630*	—	—	—	(118)	(57)	—
300	—	—	—	—	—	(35)	—	(150)
330	—	—	—	—	—	—	(56)	—
360	—	—	—	—	—	—	—	(117)

* The pressure was raised to 30 mm; figures in brackets after washing out the procaine.

TABLE 6. The effect of raising the intraluminal pressure on the release of 5-HT (ng/30 min) in the presence of hexamethonium 10^{-5} in the outside solution. Figures are means (number of observations in brackets)

Pressure...	8 mm	25 mm
Expt. 1	93 (2)	234 (3)
2	76 (4)	122 (4)
3	55 (3)	71 (3)

The effect of hexamethonium. Hexamethonium 10^{-5} was applied outside in three experiments and peristalsis was gradually abolished. At first, however, as with procaine, increased muscular activity was observed. During the continued presence of hexamethonium the intestine relaxed more and more, and it became abnormally distended when the intraluminal pressure was raised to 30 mm. At this stage, which occurred in one experiment after 4 hr, the correlation between the height of intraluminal pressure and 5-HT release was abolished. Otherwise the normal correlation was observed as shown in Table 6.

DISCUSSION

The method used in this investigation for recording the transport of fluid during peristalsis by isolated loops of intestine was developed for two reasons: in order to distinguish between peristalsis, i.e. propulsive movements, and other muscle contractions, and in order to study changes in threshold and peristaltic activity when substances were introduced into the interior of the intestine. An investigation of the action of 5-HT on peristalsis, introducing it into the lumen of the intestine, was the obvious step to be taken after evidence had been obtained (Bülbring *et al.* 1957) that the sensory receptors responsible for triggering the peristaltic reflex were situated in the intestinal mucosa. 5-HT is known to stimulate sensory endings in other parts of the body (Armstrong, Dry, Keele & Markham, 1952, 1953; Douglas & Toh, 1953; Mott & Paintal, 1953; Schneider & Yonkman, 1953; Paintal, 1954) and when it was introduced into the lumen of the intestine it stimulated peristalsis. The facilitation of the reflex when 5-HT was applied to the mucosal surface was in striking contrast to its inhibitory action (Kosterlitz & Robinson, 1957; Ginzel, 1957) when it was applied to the serosal surface. This inhibitory effect was always obtained in guinea-pig small intestine but it was not observed in rabbit small intestine. A study of different animal species will therefore be valuable in elucidating the site of action of 5-HT applied outside. It is interesting to note in this connexion that another sensory stimulant, phenyldiguanide, when introduced into the intestinal lumen, stimulated peristalsis like 5-HT. Applied outside, phenyldiguanide caused ganglion block. A detailed investigation of the action of sensory stimulants on the intestine is in progress. In rabbits the effect of 5-HT applied to the mucosa was only quantitatively, but not qualitatively different from that in guinea-pigs. Here the mechanism of action appeared to be essentially the same in both species, which is important for the conclusion reached, that 5-HT plays a physiological role in peristalsis.

The release of 5-HT into the lumen was similar in the two species; it was related to the height of the intraluminal pressure and to the peristaltic performance. In both species perfusion of the lumen with a solution containing 5-HTP greatly increased the release of 5-HT and at the same time augmented peristaltic activity. This result indicated that the threshold of intraluminal pressure required to elicit peristaltic contractions was controlled by the amount of 5-HT synthesized locally.

In isolated loops of intestine the production of 5-HT was found to decline and the threshold of intraluminal pressure to rise gradually with time. The addition to the perfusion fluid of the precursor and also of the material required for the co-enzyme of decarboxylation lowered the threshold most efficiently. Indeed, peristaltic activity was stimulated by the addition of 5-HTP and pyridoxal for longer periods than by adding 5-HT itself. Another indication of the importance of the local production of 5-HT was the result obtained with Marsilid which, presumably by maintaining the local concentration of 5-HT, had a prolonged beneficial effect on fluid transport.

When 5-HT was introduced into the lumen of a loop of intestine from which the mucosa was removed it exerted no effect whatsoever. When cocaine or procaine was present in the lumen and peristalsis was abolished, 5-HT was quite ineffective. These results suggest that the sensory endings had to be intact for the action of 5-HT and that, without the sensory organ, 5-HT could exert no action. On the other hand, it is unlikely that 5-HT has a primary function in the peristaltic reflex because, in the presence of its antagonists inside the lumen, peristalsis was never abolished. LSD and BOL raised the pressure threshold and they abolished the effect of added 5-HT; but peristalsis continued at a slower rate. Secondly, when in the course of many hours of peristalsis the release of 5-HT declined to 10 % or less, the fluid transport, though diminishing at first in parallel with the 5-HT, was maintained at 65 % of the initial volume.

Experiments were carried out in order to ascertain whether 5-HT was liberated as a result of peristaltic activity or as a result of pressure on the mucosa. Procaine was administered inside to block mucosal receptors; both procaine and hexamethonium were applied outside to produce ganglion block. While procaine produced vigorous muscle contractions of very high frequency, hexamethonium only caused a short stimulation after which contractions ceased. Both substances reduced the magnitude of the changes in 5-HT output on increasing the pressure, but they did not abolish the correlation. It may well be that muscular activity contributes to the effect of increasing the intraluminal pressure. A peristaltic wave usually causes a brief but considerable further rise in pressure, which may be much less if muscle contractions are incoordinated as in the presence of procaine. Further experiments using other substances to abolish muscular movement are required. On this point it is interesting that in one experiment the addition of eserine to the bath increased the force and efficiency of muscular contractions and at the same time increased the 5-HT output. It may be that the primary factor for 5-HT release is the pressure on the mucosa, that muscular activity itself is not an important factor, but that a secondary influence is exerted by true peristaltic contractions causing an additional rise in pressure on the mucosa.

A complicating action of procaine, when administered inside, was that the release of 5-HT was maintained at an abnormally high level. This was similar to the effect of Marsilid and presumably was due to inhibition of amine oxidase allowing an accumulation of 5-HT.

It is possible that 5-HT not only stimulates pressure receptors but also chemoreceptors. Ginzel & Kottegoda (1954) suggested that 5-HT stimulated baroreceptors as well as chemoreceptors in the carotid sinus; and since the action of acetylcholine, when applied in low concentrations to the mucosal surface of the intestine, resembled that of 5-HT, the same may be true for the small intestine.

The conclusion which may be drawn from this investigation is, therefore, that the peristaltic reflex is elicited by stimulation of receptors in the mucosa which are sensitive to pressure, and that the reflex still occurs in conditions in which only very small amounts of 5-HT are liberated or when 5-HT is ineffective, as in the presence of LSD and BOL. However, 5-HT is formed locally, probably stored locally, and released in proportion to the rise in intraluminal pressure; so that normally the peristaltic reflex is elicited at a lower intraluminal pressure because the threshold of excitation of the sensory endings is lowered by the local production of 5-HT.

SUMMARY

1. The method used for studying the peristaltic reflex in isolated loops of guinea-pig ileum and rabbit jejunum allowed the introduction of substances into the interior, so that they exerted their action on the mucosa. Changes in pressure threshold and fluid transport could thus be observed after the application of substances outside as well as inside.

2. When 5-hydroxytryptamine (5-HT) in concentrations from 10^{-9} to 10^{-5} was added to the fluid passing through the lumen of the intestine, it stimulated peristalsis. It lowered the threshold of intraluminal pressure required to elicit the reflex, it increased the frequency of contractions and it increased the volume of fluid transported.

3. Applied outside, 5-HT inhibited peristalsis in low concentrations and abolished it in high concentrations. 5-HT did not stop peristalsis in rabbit jejunum.

4. LSD (lysergic acid diethylamide) and BOL (2-brom-D-lysergic acid diethylamide) produced the opposite effect to that of 5-HT when applied inside; they inhibited peristalsis, raising the threshold, reducing the frequency of contractions and diminishing fluid transport; but they did not abolish peristalsis. However, applied outside, LSD and BOL exerted the same effect as 5-HT, inhibiting peristalsis in low concentrations and abolishing it in high concentrations.

5. 5-HT was found in the effluent after passage through the lumen of the intestine. The amount released was increased by raising the intraluminal pressure. A correlation existed between the intraluminal pressure, the release of 5-HT and the volume of fluid transported.

6. The release of 5-HT into the fluid passing through the lumen steadily decreased during several hours of peristalsis. This decline was less rapid in the presence of Marsilid (1-*iso*nicotinyl-2-*iso*propylhydrazine) which also improved the propulsive activity.

7. Addition of 5-hydroxytryptophan to the solution passing through the intestinal lumen greatly increased the amount of 5-HT in the effluent and, at

the same time, increased peristaltic activity. When pyridoxal was also present this effect was enhanced.

8. Acetylcholine and histamine applied outside in low concentrations stimulated peristalsis, in high concentrations prevented it by causing spastic contraction. Histamine had no effect inside, but acetylcholine had the same effect when applied inside as outside.

9. Phenyldiguanide applied inside caused stimulation of peristalsis followed by inhibition.

10. When the peristaltic reflex was abolished either by applying hexamethonium outside, or by applying procaine inside or outside, 5-HT was still released into the intestinal lumen and the amount was increased by raising the intraluminal pressure.

11. The observations have led to the conclusion that 5-HT, formed and stored locally in the mucous membrane, is released in proportion to the rise in intraluminal pressure and that it sensitizes pressure receptors which are situated in the mucosa.

We are indebted to Sandoz Products, Ltd., for the supply of 5-hydroxytryptamine, LSD and BOL; to Roche Products, Ltd., for supplying Marsilid; to May and Baker, Ltd., for 5-hydroxytryptophan.

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THE EFFECT OF THE LOCAL APPLICATION OF PROCAINE ON THE STRETCH REFLEX OF THE SOLEUS MUSCLE OF THE CAT DECEREBRATED BY ANAEMIA

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In 1919 Liljestrand & Magnus showed that the muscular rigidity of a decerebrate cat could be locally removed by the intramuscular injection of a quantity of procaine solution insufficient to paralyse the response of the muscle to stimulation of its nerve. They suggested that procaine paralysed the afferent nerve fibres before the motor nerve fibres. Recently (Matthews & Rushworth, 1957*a*) the same effect was produced by applying procaine solution to the nerve to the soleus muscle. This abolished the tonic stretch reflex of soleus before affecting its direct motor response to stimulation of the nerve central to the anaesthetized region. Separate experiments showed that procaine paralysed the large afferent and the large efferent nerve fibres of soleus at the same time, but that the γ efferent nerve fibres were paralysed considerably earlier (Matthews & Rushworth, 1957*b*). It was suggested, therefore, that the selective paralysis of the stretch reflex was caused by paralysis of the γ efferents supplying the muscle spindles, rather than by paralysis of the afferent pathway for the stretch reflex.

The previous experiments (Matthews & Rushworth, 1957*a*) were performed on cats decerebrated by the classical method of transecting the mid brain and removing the forebrain (Sherrington, 1898). This produces a preparation with much activity of the γ motoneurones, but little independent activity of the large α motoneurones (Eldred, Granit & Merton, 1953), and its extensor rigidity is abolished by dorsal root section (Sherrington, 1898). Cats decerebrated by the anaemic method of tying the carotid and basilar arteries (Pollock & Davis, 1930) behave differently in certain respects, probably because of additional damage to the anterior lobe of the cerebellum. First, the γ motoneurones of cats decerebrated by anaemia are in general less active than those of cats decerebrated by inter- or pre-collicular section (Granit, Holmgren & Merton (1955), using the soleus muscle). Secondly, anaemic decerebrates may remain rigid after dorsal root section (Pollock & Davis, 1930, 1931), and this does not significantly alter 'the reflex movements in soleus elicited from neck and pinna' (Granit *et al.* 1955). Thus the extensor α motoneurones of the anaemic decerebrate are kept highly excitable independently of muscle proprioceptors and the γ system. In this preparation, therefore, paralysis of the γ efferents would not be expected to abolish the stretch reflex, but only to reduce its size by a variable amount depending upon the amount of γ efferent activity in the particular cat studied. The effect of procaine on the stretch reflex of soleus has therefore been studied using cats decerebrated by anaemia instead of by brain section. In contrast to the previous results it has been found that the stretch reflex persisted until the motor nerve fibres were paralysed, as has already been briefly published (Matthews, 1957). This supports the view that the selective paralysis by procaine of the stretch reflex in cats decerebrated by brain section is due to paralysis of the γ efferents.

METHODS

The results to be described were obtained from eight cats in each of which the reflexes remained relatively constant for up to 8 hr, and in each of which procaine was applied two or more times. In all, twenty-nine applications of procaine were made. The methods of Matthews & Rushworth (1957*a*) were closely followed. In addition the recording of muscle tension was supplemented by recording the electrical activity of soleus. The methods, avoiding repetition of detail previously described, were as follows.

Preparation. The cats were anaesthetized with ether and then decerebrated by the anaemic method of tying the carotid and basilar arteries (Pollock & Davis, 1923, 1930). The basilar artery was exposed by retracting the pharynx to one side, removing the pre-occipital muscles and nibbling away the base of the skull from the foramen magnum upwards. After reflexion of the dura mater the basilar artery was ligated, just above (rostral to) the inferior cerebellar arteries (cf. Fig. 1, Pollock & Davis, 1923). Each common carotid artery was then ligated, both low in the neck and just below the internal carotid artery. Each external carotid artery was ligated, together with the lingual artery, at the bifurcation. The internal carotid, occipital and ascending pharyngeal arteries, which arise from the carotid close together (Davis & Story, 1943), were ligated with a single thread. These three vessels together probably correspond to the 'internal carotid artery' ligated by Pollock & Davis (1930). In the cat the internal carotid artery has no lumen and carries no blood to the brain, while the occipital and the ascending pharyngeal arteries both supply the brain (Davis & Story, 1943; Chungcharoen, Daly, Neil & Schweitzer, 1952).

Shortly afterwards an inter-collicular section was made, and the forebrain was removed. The rest of the dissection was performed without further anaesthetic. It was often necessary to ventilate the cats artificially for a period after the anaemic decerebration. In three cats this was continued throughout the experiment as their spontaneous breathing was apneustic and appeared inadequate; they retained reflex rigidity throughout.

On the right side the nerve to soleus and lateral head of gastrocnemius was separated from the sciatic, and the rest of the sciatic was cut. The remaining major nerves supplying the hind limb were also cut or ligated. The right soleus was separated from gastrocnemius and was attached to an isometric myograph. In four experiments in which the electrical activity of soleus was recorded, the nerve to soleus was dissected out of the lateral head of gastrocnemius through which it runs, and all branches to gastrocnemius were cut. This ensured that the electrical activity recorded arose from soleus alone.

Recording. The tension in soleus was recorded with a myograph which, with its supports, had a yield of 0·1 mm/kg. To elicit the stretch reflex the myograph could be moved back to a fixed stop either by hand or, at a slower and more constant rate, by a screw thread turned by an electric motor. An extension of 13 mm was most frequently used.

The electrical activity in soleus was recorded between the insulated tendon hook and a thin plate of silver foil lying on the belly of soleus. The preparation was earthed by another silver plate embedded in the thigh muscles. The electrodes were connected to a push-pull amplifier whose frequency response was flat from 50 c/s to 1·5 kc/s. The output of the amplifier and that of the myograph were displayed on the two beams of a double cathode-ray tube, and photographed on slowly moving paper.

Stimulation. The same stimulator was used both to test the functional continuity of the motor fibres to soleus, and to inhibit the stretch reflex by stimulating the lateral popliteal nerve of the same side. It provided approximately exponential pulses (0·5 msec time constant, derived from a blocking oscillator) of controllable frequency.

Procaine. Neutralized procaine solution of 0·2–0·5 % was freshly made up for each experiment. A 1·5 × 1·5 cm square of filter paper was soaked in the procaine solution, bent into a V, and placed over the common nerve to soleus and lateral head of gastrocnemius as it lay in a pool of liquid paraffin.

RESULTS

After anaemic decerebration marked extensor rigidity developed in all limbs and persisted throughout the experiment. On connecting soleus to the myograph, 'active tension' was developed at lengths corresponding approximately to its normal length, and the tension fell when the soleus motoneurones were inhibited by stimulating the lateral popliteal nerve of the same side. Even when soleus was disconnected from the myograph and was left slack, muscle action potentials could still be recorded from it. The initial length of soleus was set so that the 'active' resting tension was less than about 50 g, and soleus was thereby probably arranged so as to be below its resting length in the body. When the active tension was inhibited or paralysed by procaine, soleus became quite slack at the initial length and developed only a few grams of resting tension.

On extending soleus by 13 mm a stretch reflex of up to 1400 g was found, over and above the passive increase in tension produced by the same extension. The tonic stretch reflex was well marked, and the reflex tension showed little tendency to decrease from its initial level immediately after extending soleus. The initial length of soleus used was below the optimum length for developing tension in a twitch produced by a single stimulus to the nerve to soleus. When soleus was extended by 13 mm the twitch tension increased several-fold from its initial value of 100 g or less. It still did not reach a maximum. When soleus was extended the twitch was often obscured by a reflex contraction superimposed upon it, probably as a result of stimulating afferent fibres in the nerve. The tension produced in soleus by stimulating its nerve at 90/sec was much less affected by extending soleus, and was only increased by 15–60 % by a 13 mm extension. The tendon jerk produced by tapping on the tendon hook was very small, and was sometimes undetectable (under 5 g). This appeared to be partly because of the short length of soleus, and partly because of occlusion of the jerk by the constant resting discharge. When soleus was extended the jerk did not usually increase but was occluded by the stretch reflex. Thus in the anaemic decerebrate it has not been feasible to study the jerk and the twitch quantitatively, as was previously done in cats decerebrated by section (Matthews & Rushworth, 1957*a*). There soleus could be extended to a greater length (as judged by the passive tension) without exciting a resting discharge, and the jerk and the twitch were larger and more constant. In the present experiments the main interest has been to compare the relative effects of procaine on the stretch reflex and on the motor tetanus.

The persistence of the stretch reflex until the paralysis of the tetanus

When procaine solution was applied to the nerve to soleus, the stretch reflex survived as long as the motor tetanus elicited by stimulation of the nerve central to the anaesthetized region. This is shown in the records of Fig. 1, taken from a typical experiment. Fig. 1*a* shows the stretch reflex produced by suddenly extending soleus by 13 mm and keeping it extended for 4 sec. The amount of active tension present is shown by inhibiting the soleus motoneurones. This was done by stimulating the lateral popliteal nerve of the same side for the period indicated by the signal marker beneath the tension record. The active tension was about 300 g during the stretch, while it was under 20 g at the resting length of soleus, as shown by the second period of inhibition in Fig. 1*a*. Fig. 1*f* shows the motor tetanus elicited, before applying procaine, by stimulating the nerve at 90/sec. The rest of Fig. 1 shows the progressive change in the stretch reflex and the tetanus after applying 0·3% procaine solution to the nerve peripheral to the stimulating electrodes, and the recovery after removing the procaine. Eleven minutes after applying procaine the tetanus was reduced to half (Fig. 1*g*) and the stretch reflex was also reduced (Fig. 1*b*). After 14 min the response to stimulation at 90/sec had just disappeared (Fig. 1*h*), but the stretch reflex was still present (Fig. 1*c*) and was not paralysed until later (Fig. 1*d*). On removing the procaine and covering the nerve in Ringer's solution, both the stretch reflex and the tetanus returned to their original size. Fig. 2 presents the results of this experiment graphically, and again shows clearly that the stretch reflex survived until slightly after the tetanic response had been abolished. This may seem surprising, but the disappearance of the tetanic response is partly due to Wedensky inhibition of nerve fibres which are still able to conduct impulses at a lower frequency than 90/sec, and can therefore mediate the stretch reflex. The responses of soleus to stimulation of its nerve by single shocks and at lower frequencies than 90/sec also persisted longer than the response to stimulation at 90/sec. Thus the motor tetanus at 90/sec only roughly indicates when the motor nerve fibres are being paralysed, and it is not a quantitative measure of the degree of

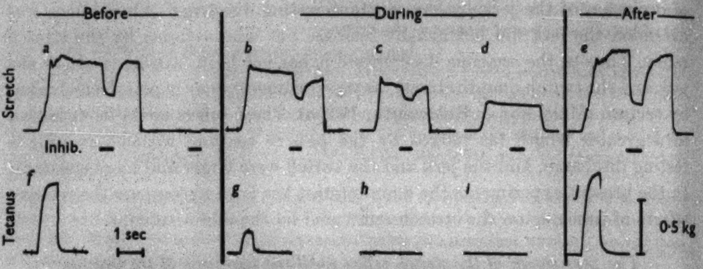

Fig. 1. Progressive paralysis by procaine of the stretch reflex and motor tetanus. Myographic records of the stretch reflex elicited by extending soleus 13 mm (above), and of the tetanus produced by stimulating its nerve at 90/sec (below). During the signal (line below stretch records) the stretch reflex was inhibited by stimulating the lateral popliteal nerve at 90/sec. Records were taken at various times after the application of 0·3% procaine to the nerve: *a* and *f*, 6 min before; *b* and *g*, 11 min after; *c* and *h*, 14 min after; *d* and *i*, 29 min after; *e* and *j*, 22 min after removing procaine. (Records retouched by removing electromyogram record which lay immediately beneath tension record; in a few places the records overlapped and were not then separated. The passive tension decreased slightly during the experiment.)

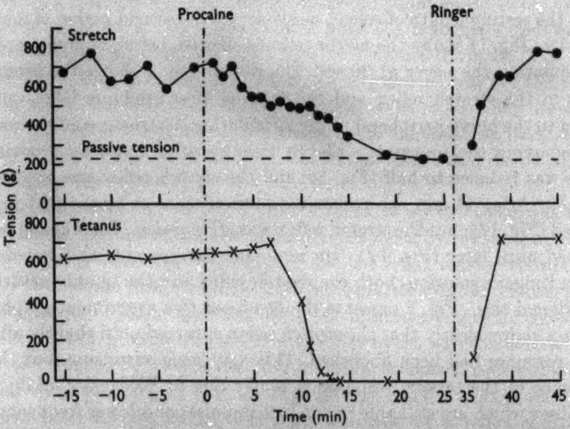

Fig. 2. A graph of the experiment illustrated in Fig. 1. Stretch tension (above) and tetanus tension (below) plotted against time: the increase in tension produced by stretching was measured in each case 1·5 sec after the beginning of a 13 mm extension; passive tension similarly determined when the reflex was paralysed; tetanus tension measured 0·5 sec from beginning of contraction. The stretch reflex persisted slightly longer than the tetanus elicited by stimulation of the nerve at 90/sec.

functional paralysis of the motor fibres. A lower frequency of stimulation might provide a better criterion of the degree of functional paralysis of the motor fibres but would then be less applicable to the afferent fibres. The motor tetanus at 90/sec does provide, however, an arbitrary measure of the degree of paralysis of the nerve which enables the present experiments to be compared with the previous experiments on cats decerebrated by the classical method. There the motor tetanus was elicited by stimulation at 100/sec, but, in marked contrast to the present experiments, the tonic stretch reflex was paralysed long before the tetanus. The cause of the paralysis of the stretch reflex by procaine must, therefore, be different in the two preparations. In the present experiments the obvious cause for the disappearance of the stretch reflex is the paralysis of the direct stretch reflex pathways (afferent and efferent); in the cats decerebrated by section this, then, cannot be the cause, and selective paralysis of the γ efferents seems the likely explanation.

The biphasic paralysis of the stretch reflex

After anaemic decerebration there is some activity of the γ motoneurones supplying the ankle extensors (Granit *et al.* 1955). Paralysis of the γ efferents should, therefore, decrease the size of the stretch reflex, for extension of soleus would then excite a smaller afferent discharge in the large fibres of the muscle spindles. Procaine does paralyse the γ efferents of soleus considerably earlier than the large fibres from soleus (Matthews & Rushworth, 1957*b*); therefore in the present experiments some diminution of the stretch reflex would be expected to occur before the motor tetanus is altered. Such a selective effect on the stretch reflex was usually produced; it occurred soon after the application of procaine, and can be seen in Fig. 2. Here, 5 min after applying procaine the stretch reflex had decreased by 100–200 g from its previous level, but the tetanus was unaltered or slightly increased. Over the next 6 min the stretch reflex decreased by only about a further 50 g, while the tetanus decreased to a third of its original value. Over the next 8 min both were abolished.

A similar small initial decrease in the stretch reflex, occurring at a time when the motor tetanus was unaltered, was found following each of the three other applications of procaine in this cat; in each case the change was little larger than the spontaneous changes in the stretch reflex in this cat, as in Fig. 2. Fig. 3 shows a graph from an experiment on another cat in which the early change was much greater. Here there is no doubt that the paralysis of the stretch reflex occurs in two stages, an early stage when the tetanus is unaltered and a later stage concurrent with the paralysis of the tetanus. Fig. 4 was obtained from yet another cat, and here no early change in the stretch reflex is detectable (the slight increase is too small to be significant). These three graphs (Figs. 2–4) illustrate the results obtained in different cats. In any one cat the relative magnitudes of the first and second stages of the paralysis of the reflex were more constant. Results similar to that shown in Fig. 2 were most common. In only one instance out of twenty-nine applications of procaine was the stretch reflex completely paralysed at an early stage, as had usually been found in cats decerebrated by section (upon an earlier application of procaine in this cat the initial paralysis was not quite complete). In seven other cats (twenty-seven applications of procaine) the stretch reflex did not disappear until the motor tetanus was being paralysed. Sometimes the reflex

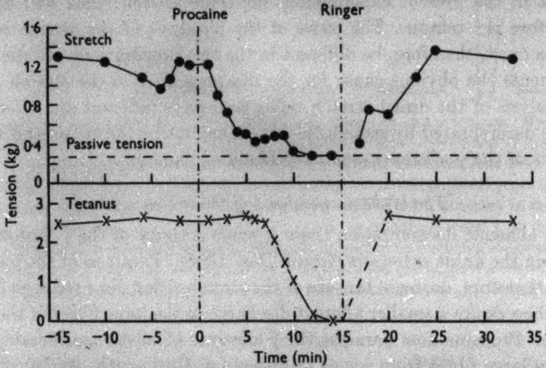

Fig. 3. Pronounced biphasic paralysis of the stretch reflex in a different cat. A graph of the stretch tension and tetanus tension plotted against time; stretch tension measured 1·5 sec after the beginning of a 13 mm extension; tetanus at 90/sec; 0·25% procaine used. (Tension scale in error, each value should be multiplied by 0·63 to give the correct tension.)

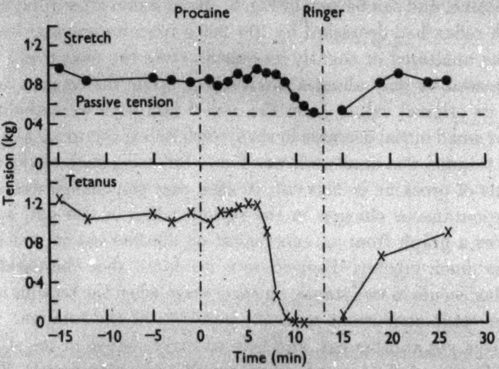

Fig. 4. No detectable initial paralysis of stretch reflex in another cat. A graph of stretch tension and tetanus tension plotted against time; each stretch tension measured 1 sec after beginning of a 6 mm extension; tetanus at 90/sec; 0·2% procaine used.

disappeared while the tetanus elicited by stimulation at 90/sec was not completely paralysed (Fig. 3); sometimes the reflex survived slightly longer than the tetanus (Figs. 2, 4). The final complete paralysis of the reflex usually preceded that of the tetanus in experiments in which the initial partial paralysis had been marked and the remaining reflex small.

In cats decerebrated by brain section a biphasic paralysis of the stretch reflex was found very occasionally, but usually the tonic stretch reflex was abolished well before the tetanus at 100/sec (Matthews & Rushworth, 1957*a*). The two phases of paralysis of the stretch reflex occur at distinctly different times and cannot have the same cause. The second, final, phase of paralysis is presumably due to paralysis of the direct afferent and efferent pathways for the stretch reflex. The first phase can only simply be explained by the selective paralysis of smaller nerve fibres, and the γ efferents are the only ones known whose paralysis would have this effect. The variability of the magnitude of the first phase would then be caused by a variable degree of γ motoneurone activity from cat to cat.

Electromyographic confirmation of the persistence of the stretch reflex

It is conceivable that in the experiments so far described the 'stretch reflex' which persisted so long was not reflex at all. In anaemic decerebrates there is a constant resting discharge from the large α motoneurones. This constant discharge probably produces more tension in the stretched than in the unstretched soleus, for the tetanus tension is increased by lengthening soleus. From the myogram alone this increase in active tension on stretching would appear to be due to a stretch reflex. The two phases of paralysis might perhaps then be interpreted as due to an early paralysis of afferent fibres and a later paralysis of motor fibres. This is unlikely, but it seemed best to exclude it directly by electromyography.

In six cats, recording leads were placed on the belly and on the tendon of soleus and the electromyogram was recorded simultaneously with the myogram. In four of these cats the lateral head of gastrocnemius was denervated to ensure that the potentials recorded arose solely from soleus. (In purely myographic experiments the delicate separation of the nerve to soleus from the nerve to the lateral head of gastrocnemius was not performed. Mechanical isolation of soleus was obtained by attaching it alone to the myograph.) At the initial lengths of soleus used, a considerable amount of electrical activity was usually detected (0·5–1 mV, peak to peak). When soleus was stretched the electrical activity increased. This electrical activity appeared to be recorded solely from soleus, for it disappeared when the nerve to soleus was cut or completely paralysed with procaine. It also disappeared when soleus was inhibited by stimulating the lateral popliteal nerve. When procaine was applied to the nerve to soleus the amount of electrical activity, both at rest and on stretching, decreased at roughly the same rate as the myographically determined stretch reflex. As the amount of electrical activity decreased it became possible to recognize uniform-sized potentials occurring regularly, which were presumed to be due to the discharge of single motor units. Finally, only a few motor units remained active, and it was then usually found that their frequency of firing increased on stretching, thereby showing that the stretch reflex was still present. By this criterion it was fully confirmed that the stretch reflex persisted as long as or slightly longer than the tetanus elicited by stimulation of the nerve at 90/sec.

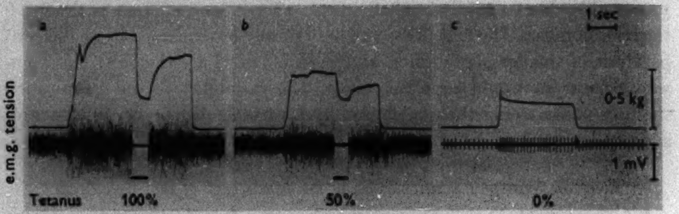

Fig. 5. Simultaneous myographic and electromyographic records obtained from soleus during progressive paralysis of its nerve with procaine. Above, the tension produced in soleus by a 13 mm extension; below, the electromyogram recorded between belly and tendon of soleus; during the signal (below e.m.g. records) the stretch reflex was inhibited. The size of the tetanic response is shown below each record. In *c* the electrical discharge has been reduced to that from a single motor unit which is still influenced by stretching soleus.

Fig. 5 shows the changes in the electromyogram produced by the application of procaine. The electrical record lies immediately below the tension record. Fig. 5*a*, obtained before the application of procaine, shows the discharge at the initial length of soleus and its increase on suddenly stretching soleus. During the signal marker soleus was inhibited and its electrical activity ceased. Fig. 5*b* shows these responses at a time when the tetanus (at 90/sec) was half paralysed by procaine. Fig. 5*c* was taken shortly after the tetanus had been abolished. There is only a single motor unit active, but its frequency of discharge increased when soleus was stretched. Clearly it was still reflexly excited by the discharges from soleus proprioceptors, and the stretch reflex was therefore still just present. The size of the potential recorded from this single motor unit increased when soleus was stretched, probably because the inter-electrode distance then increased. This was a common finding with other single units, and it complicated the interpretation of the records obtained when several units were active. This difficulty was largely overcome when soleus was stretched slowly instead of suddenly, by moving the myograph with a motor-driven screw thread instead of by hand. The discharges of several different motor units could then be more readily followed during stretching in spite of changes in the size of their recorded potentials.

Fig. 6, from a different experiment, shows the electromyogram and the myogram obtained on slowly extending soleus by 13 mm (start at *A*, stop at *B*) and then slowly releasing soleus (*C* to end). In Fig. 6*a*, taken after applying procaine, the tetanus (at 90/sec) had been reduced to one half and the stretch reflex to one third of their original values. On releasing soleus the discharge ceased temporarily, presumably from a slowing of the discharge from muscle spindles to below their resting rate. In Fig. 6*b*, taken when the tetanus had just disappeared, the resting discharge in the electromyogram had been abolished; but there was still a stretch reflex involving several motor units. Fig. 6*c* was taken 2 min later when only a single motor unit was excited by stretching soleus, thus showing again that the stretch reflex persisted longer than the tetanus at 90/sec.

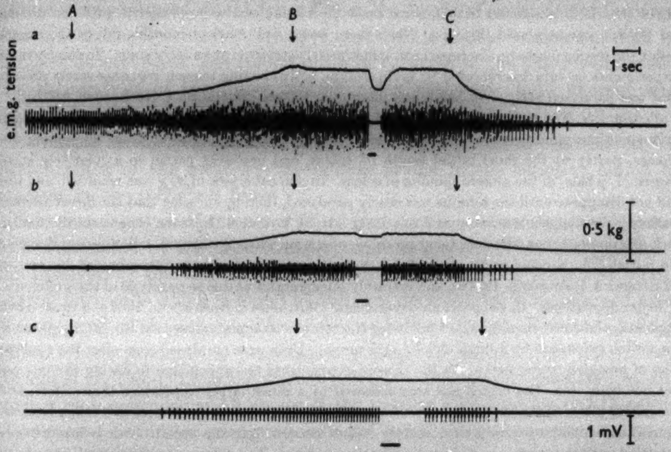

Fig. 6. The myographic and electromyographic responses of soleus to slow stretch and release at three different times after the application of procaine to its nerve. In each record an extension of 13 mm starts at *A* and stops at *B*; release starts at *C* and continues to the end of the record. Rate of extension, 1·6 mm/sec, release slightly faster; stretch reflex inhibited during signal (below e.m.g. records). In *a* the tetanus was reduced to half its original size, in *b* and *c* it had been abolished. In *c* only a single motor unit is excited on stretching soleus.

By this method of observing the discharge of the few motor units remaining active at the time when the nerve was nearly completely paralysed, it has been repeatedly confirmed that the stretch reflex persists as long as the motor tetanus elicited by stimulation at 90/sec. Sometimes when a resting discharge persisted after the tetanus at 90/sec had been abolished, it was found that the discharge of some units was not influenced by stretching soleus. Presumably the soleus motoneurones were being strongly excited by central pathways and the partially paralysed afferent discharge from soleus proprioceptors was not sufficient to influence their discharge.

Resting tension. About 50 g of active tension was usually developed at the initial lengths of soleus used. This active tension could be detected myographically, either by inhibiting the soleus motoneurones, or by observing small irregularities in the tension record (up to 10 or 20 g) produced by the slightly irregular contraction of soleus. The resting discharge of soleus motoneurones was, however, more readily determined from the electromyographic record. By each of these three criteria it was found that in some cases the resting discharge persisted until the tetanus (at 90/sec) was paralysed (Fig. 5*c*). When there was a well marked biphasic fall of the stretch reflex the resting discharge decreased and sometimes disappeared at an early stage. In the previous experiments on cats decerebrated by brain section, active resting tension was only rarely present and was paralysed early, well before the tetanus at 100/sec (Matthews & Rushworth, 1957*a*).

Tendon jerk. The tendon jerk, produced by tapping on the tendon hook, has usually been small and sometimes undetectable. This appeared to be due partly to occlusion from the resting discharge, partly to the short initial length of soleus, and probably partly to a relatively small degree of 'γ bias' of the muscle spindles of soleus. In one cat a jerk of 30 g was recorded, and this did not disappear until the tetanus was nearly paralysed, thereby showing that the direct afferent pathway for the jerk was paralysed relatively late by procaine. In three other cats the tendon jerk disappeared soon after applying procaine, at a time when the stretch reflex was undergoing its initial fall. Similar early paralysis of the jerk was found in cats decerebrated by section (Matthews & Rushworth, 1957*a*), and is readily interpretable as due to paralysis of the γ efferents.

Reflex appendages. In the previous experiments (Matthews & Rushworth, 1957*a*) a small reflex appendage was occasionally found following the tetanus or superimposed on the falling phase of the twitch (produced by a single shock to the nerve). Both were paralysed soon after the application of procaine to the nerve. In the present experiments the appendage following the tetanus has not been seen. The twitch has been followed by a much larger appendage than before, and the falling phase of the twitch has often been completely obscured by an irregular reflex contraction which sometimes produced a slightly higher tension than the twitch. This reflex response persisted until the nerve was almost completely paralysed. The difference in the sensitivity of the 'twitch appendage' to procaine between the two preparations would be expected, for reflex contractions appear to be dependent on the γ efferents in cats decerebrated by section but not in those decerebrated by anaemia (Eldred *et al.* 1953; Granit *et al.* 1955).

Motor responses. In each experiment it was confirmed that the threshold of the motor fibres was not altered during the progressive paralysis of the nerve by procaine applied 1 cm away from the stimulating electrodes. It was also confirmed that the stimulus remained maximal throughout. The twitch was very inconstant in size and could not be used as an accurate measure of the number of conducting motor fibres. The tetanus varied slightly in size, and often was increased for a time after the nerve had recovered from paralysis. It is possible that in cats with so much motor discharge there is a variable amount of neuromuscular block or of muscle fatigue. When the twitch was recorded it was found to be paralysed only slightly after the tetanus, as had been found before (Matthews & Rushworth, 1957*a*).

DISCUSSION

The action of procaine on the stretch reflex of cats decerebrated by anaemia differs markedly from its action in cats decerebrated by section. The difference, however, is quantitative rather than qualitative; for in both preparations two phases of reflex paralysis have been found, an early phase while the motor tetanus (at 90–100/sec) was unaltered, and a late phase while the tetanus was being paralysed. In cats decerebrated by section the tonic stretch reflex was usually completely paralysed in the early phase. In cats decerebrated by anaemia the stretch reflex persisted until the late phase, and the early change was usually small. The difference between the two preparations would be expected if the first phase of the paralysis of the stretch reflex is due to a selective paralysis of the γ efferents. Its magnitude would then depend upon the amount of γ motoneurone activity, which is higher in ordinary than in anaemic decerebrates. Whether or not the stretch reflex persisted after the γ fibres were paralysed would depend upon the degree of excitation of the large α motoneurones by central pathways, and this is higher in anaemic than in ordinary decerebrates. The second phase of paralysis of the reflex is obviously due to paralysis of the direct stretch reflex pathways, both afferent and efferent, and this cannot be the cause of the early phase of paralysis. Thus these experiments strengthen the view that the selective action of procaine on the stretch reflex of the ordinary decerebrate is due to the selective action of procaine on the γ efferent fibres.

Selective paralysis of the nerve fibres from 'tonic α motoneurones' appears to be eliminated by the present experiments as a cause of the early phase of the paralysis of the stretch reflex. The difference between tonic and phasic ventral horn cells which is found in ordinary decerebrates is maintained after decortication of the anterior lobe of the cerebellum (Granit, Henatsch & Steg, 1956), which produces a preparation similar to the anaemic decerebrate (Granit *et al.* 1955). Thus the stretch reflex was presumably mediated by the tonic α motoneurones at all stages of the present experiments, and therefore these cannot have been paralysed until the motor tetanus was also paralysed.

The small afferent nerve fibres from muscle spindles would appear unlikely to have a great effect on the stretch reflex in this preparation. Occasionally the stretch reflex was not significantly altered until the motor tetanus was being paralysed, whereas the small afferents would be expected to be paralysed earlier. Small changes in the stretch reflex could not reliably be detected because of the spontaneous variations in tone.

The local application of procaine to nerve thus appears to block the control of muscle by the γ route considerably before it blocks excitation by the α route. Intramuscular injection of procaine probably acts in a similar way (cf. Liljestrand & Magnus, 1919). Either method should prove useful in deciding the relative importance of these two routes of muscular control in various muscular acts in both animals and man.

SUMMARY

1. Procaine solution has been applied to the nerve to the soleus muscle of cats decerebrated by anaemia, and the consequent progressive paralysis of the stretch reflex has been observed.

2. The stretch reflex survived as long as the motor tetanus elicited by stimulation of the nerve central to the anaesthetized region at 90/sec.

3. Electromyography has confirmed that the stretch reflex recorded myographically was a true reflex, and not due to a constant motor discharge.

4. The stretch reflex was usually paralysed in two stages, the initial partial paralysis occurring at a time when the tetanic response was unaltered. This early stage of paralysis is thought to be due to paralysis of the γ efferents. The later complete paralysis is due to block of direct stretch reflex pathways, both afferent and efferent.

5. The results contrast with those previously obtained on cats decerebrated by mid-brain section, where the tonic stretch reflex was usually paralysed early. The difference between the two preparations can be explained by the different activities of their α and γ motoneurones.

I should like to thank Dr C. G. Phillips for helpful discussion.

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THE DISCHARGE FROM MUSCLE SPINDLES AS AN INDICATOR OF γ EFFERENT PARALYSIS BY PROCAINE

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In a previous paper (Matthews & Rushworth, 1957*b*) it was shown that the γ motor fibres are more sensitive to procaine than either the large α motor fibres or the large muscle afferent nerve fibres. These results were obtained by recording the compound action potentials set up in the dorsal and ventral roots in response to a shock applied to a muscle nerve, and observing the changes produced by applying procaine to the nerve. In the present experiments the discharge in a single afferent nerve fibre from a muscle spindle has been used instead as an indicator of both γ fibre and afferent fibre integrity (cf. Eldred, Granit & Merton, 1953). In this way the degree of paralysis produced by procaine is tested by asynchronous discharges at natural frequencies instead of by a volley that is artificially synchronous in all nerve fibres. Thereby the previous results have been confirmed, and may now be applied more confidently to experiments on the selective alteration of the stretch reflex produced by procaine (Matthews & Rushworth, 1957*a*; Matthews, 1958).

METHODS

The present results have been obtained from nine gastrocnemius-soleus muscle spindles in eight decerebrate cats. Decerebration was effected by dividing the mid brain at intercollicular level by suction, and then destroying the forebrain. Before decerebration the right hind limb was denervated except for the nerves to the medial and lateral heads of gastrocnemius, and a lumbar laminectomy was performed. Except for the initial induction of anaesthesia with ether, the operation was performed under Thiogenal anaesthesia (Merck, Darmstadt; sodium salt of 5-(2-methylthioethyl)-5-(L-methylbutyl)-2-thiobarbituric acid). This was discontinued after decerebration.

The Achilles tendon was connected to an isometric myograph which could be moved by hand so as to stretch the gastrocnemius and soleus muscles. The discharges of single muscle spindles were obtained by subdividing dorsal root filaments (L 7 or S 1). The amplified action potentials were displayed on a cathode-ray oscilloscope and the stationary spot photographed on moving paper. Exposed roots and nerves were immersed in pools of liquid paraffin. Neutralized procaine solution (0·2–0·5 %) was applied to the nerves to gastrocnemius (the nerve to soleus runs with these) on a piece of filter paper. The degree of paralysis of the motor nerve fibres was assessed by stimulating the gastrocnemius nerves (at 90–100/sec) central to the anaesthetized region and recording muscle tension on the second beam of the oscilloscope. Further details have been given previously (Matthews & Rushworth, 1957*a*).

In each experiment the unit studied was shown to be a muscle spindle by observing the pause in its discharge during a muscle twitch produced by a single shock to the gastrocnemius nerves (B. H. C. Matthews, 1933). At the end of some experiments the nerves to the gastrocnemius and soleus muscles were cut separately to attempt to identify the muscle of origin of the spindle discharge. The conduction velocity of the fibre studied was usually roughly calculated by recording the conduction time from the stimulating to the recording electrodes and estimating the conduction distance. The velocities range from 60 to 120 m/sec, and the fibres studied therefore presumably originated in the primary ending of the muscle spindle (Hunt, 1954).

RESULTS

The clear result of these experiments has been that the discharge of a muscle spindle, recorded from a dorsal root filament, is affected in two stages by the application of procaine to the nerve supplying its muscle. First, the frequency of discharge of the muscle spindle falls suddenly to a level similar to that found after ventral root section. The frequency of discharge then remains constant at this new level, and it appears that the γ fibres have been paralysed. Secondly, the spindle afferent itself is affected, and for a short time before its complete paralysis it will not transmit high-frequency discharges (Wedensky inhibition). A single experiment demonstrating this behaviour is illustrated in Figs. 1 and 2.

Fig. 1*a* shows the discharge of a muscle spindle from the medial head of gastrocnemius (lower trace) and the tension in the Achilles tendon (upper trace). The record was taken before applying procaine and the ventral roots were intact. In the middle of the record gastrocnemius was stretched 10 mm and the new length maintained. At the initial length (tension under 50 g) the muscle spindle was discharging at 37/sec; on stretching, the rate increased and 1 sec after the beginning of the extension it was 98/sec. These discharge rates were due not only to the applied tension but also to the tonic excitation of the muscle spindle by its γ motor fibres, for when at the end of the experiment the L 6–S 2 ventral roots were cut, the discharge decreased to that shown in Fig. 1*e*. The de-efferented spindle discharged at 12/sec at the resting length, and 1 sec after stretching it discharged at only 36/sec. When procaine was applied to the nerves to gastrocnemius a similar slowing in the discharge of the muscle spindle was produced. Fig. 1*b* was obtained 10 min after applying procaine. The discharge at rest is 12/sec, and that 1 sec after stretching is 38/sec, as when the spindle was de-efferented. This slowing occurred over about 3 min, and thereafter the discharge remained constant at the de-efferented level for 13 min. The spindle nerve fibre then began to be noticeably affected by the procaine, and appeared to be unable to transmit high-frequency discharges though still transmitting low-frequency discharges (Wedensky inhibition). This is shown in Fig. 1*c*, where the resting discharge continued unchanged (12/sec). However, the discharge ceased temporarily at the stage of stretching at which there had previously been the highest rate of discharge. One minute later the afferent fibre studied was completely paralysed. When the procaine was removed and the nerve washed in Ringer's solution the original discharge pattern returned (Fig. 1*d*).

The time relationships of this experiment are illustrated as a graph in Fig. 2. For 24 min before applying procaine there was little spontaneous fluctuation of the discharge of the muscle spindle, either at rest or on stretching. However, 5 min after applying procaine the discharge fell to a new level, which was that of the de-efferented spindle. This level was then maintained nearly constant until the muscle spindle fibre itself was paralysed. The recovery was similarly step-like. In the lower part of the graph is plotted the tetanus tension produced by stimulating the gastrocnemius nerves at 90/sec, central to the anaesthetized region. At the time of the initial change in the muscle spindle

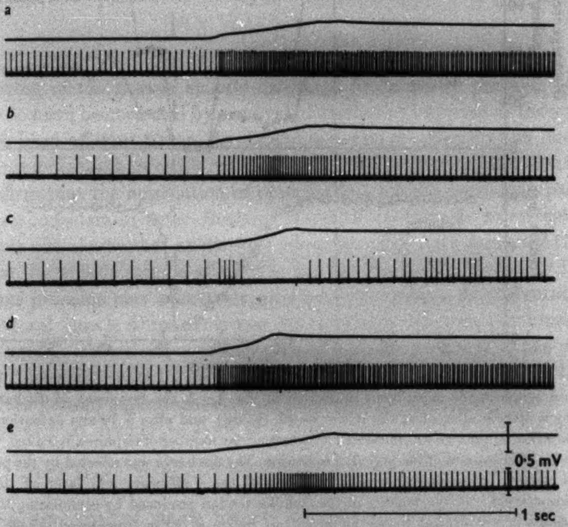

Fig. 1. Alterations in the discharge of a single muscle spindle afferent produced by applying 0·5% procaine to the muscle nerve. In each record, muscle tension above, spindle discharge below. Half way through each record the muscle was stretched 10 mm and the extension maintained. Records taken at the following times: *a*, 8 min before applying procaine; *b*, 10 min after applying procaine, γ efferents paralysed; *c*, 21 min after applying procaine, Wedensky inhibition of afferent fibre; *d*, 16 min after removing procaine, afferent fibre and γ efferents recovered; *e*, 4 hr later, after cutting ventral roots. The spindle was in the medial head of gastrocnemius. The height of the spike fluctuates slightly because it is superimposed on an irregular base line. The spike became smaller after cutting the ventral roots because the recording electrodes were moved.

discharge there was no change in the tetanus. At the time when the muscle spindle afferent was paralysed the tetanus was reduced to 25 % of its initial size. Thus in relation to the motor fibres (tested at 90/sec) the γ fibres were much more sensitive to procaine.

This muscle spindle was studied during two further applications of procaine with the same results. In one of these cases the motor fibres were tested by single shocks instead of by stimulation at 90/sec. The early failure of the γ fibres was not, therefore, due to 'fatigue' caused by the applied stimulation.

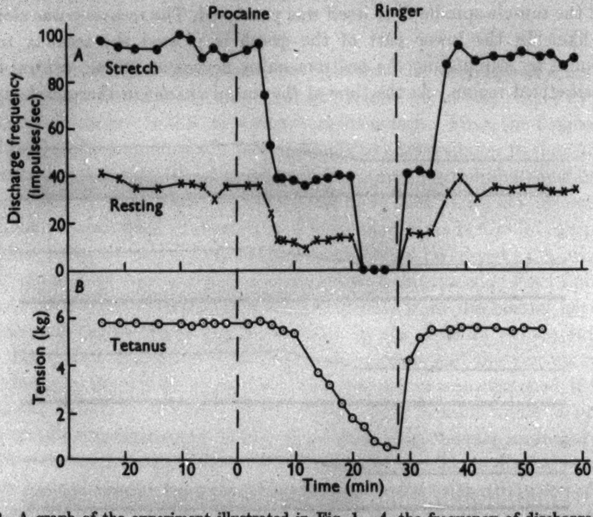

Fig. 2. A graph of the experiment illustrated in Fig. 1. *A*, the frequency of discharge of the muscle spindle afferent at the initial length (below), and after a 10 mm extension (above). The stretch discharge was measured by counting the number of impulses in 0·5 sec, beginning 0·5 sec after the end of the period of extension. The discharge was affected by the procaine in two stages, corresponding to paralysis first of the γ efferents and later of the spindle afferent itself. *B*, the tension in the Achilles tendon produced by stimulating the gastrocnemius nerves central to the anaesthetized region at 90/sec (measured 0·5 sec after the beginning of the stimulation).

Similar results have been obtained from seven other spindles. In two of these there was a change in the resting discharge on applying procaine, but not in the discharge produced on stretching; perhaps the stretch completely inhibited the γ motoneurones of these spindles (cf. Hunt, 1951; Eldred *et al.* 1953). Another spindle was apparently not being driven by its γ fibres at all. In three spindles the frequency of discharge did not at once settle to a steady level after the spindle had apparently been de-efferented by procaine. In these cases the frequency of discharge fell, as usual, soon after applying procaine; but it then increased again slightly over the next few minutes though not to its previous level. Unfortunately section of the ventral roots was only successfully completed in half the experiments, and these possibly anomalous effects have not been analysed. The general conclusion was, however, clear; the γ fibres were paralysed long before the motor tetanus (at 90–100/sec), but the spindle afferent nerve fibres were paralysed while the tetanus was being paralysed.

Sometimes after stimulating the gastrocnemius nerves a great increase in the resting discharge of the muscle spindle occurred. The stimulus was sufficiently strong to excite both the γ fibres and the smaller afferent nerve fibres, and it is not certain whether the effect on the spindle was reflex or a direct result of stimulating its γ fibres. At any rate this effect disappeared shortly after applying procaine and appeared to be dependent upon γ fibre integrity.

DISCUSSION

The slowing of the muscle spindle discharge produced by procaine is very unlikely to have been caused by removal of excitatory impulses to the γ motoneurones from afferent fibres. In decerebrate cats de-afferentation does not alter the spindle discharge (Eldred *et al.* 1953). The present experiments therefore confirm that the application of procaine to a muscle nerve may paralyse the γ fibres considerably before the large afferent fibres from the muscle spindles. They have also shown that at the time when the γ fibres are paralysed the discharge in the muscle spindle afferent is not interrupted by Wedensky inhibition. Thus procaine may selectively produce a reversible γ de-efferentation of the intrafusal muscle fibres. This was the conclusion we drew previously and used to explain the selective changes which may be produced in the stretch reflex of decerebrate cats by procaine (Matthews & Rushworth, 1957*a*, *b*). The value of the present experiments is that the degree of nerve block was tested by the physiological discharges both of γ fibres and of muscle spindle afferent fibres.

SUMMARY

1. The discharge of single muscle spindles in the gastrocnemius-soleus muscle of decerebrate cats has been studied during the progressive paralysis of the muscle's nerve by procaine. The discharge was recorded from a dorsal root filament, while the ventral roots were intact.

2. Soon after applying procaine the frequency of discharge, both at the initial length and on stretching, fell to a new level and then remained constant until the single afferent fibre studied was itself paralysed. This rate was the same as that found after cutting the ventral roots, showing that the slowing was produced by paralysis of the γ efferents to the muscle spindle.

3. At the time when the γ efferents were paralysed there was no change in the response of the muscle to stimulation of its nerve, central to the anaesthetized region, at 90–100/sec.

4. This confirms previous work showing that the γ efferents are more sensitive to the local application of procaine than either the large afferent or the large efferent nerve fibres.

We should like to thank Dr C. G. Phillips for helpful advice.

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MOTOR UNITS IN THE RAT DIAPHRAGM

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The electrical 'units' recorded in electromyographic studies are commonly assumed to represent the activity of motor units (Smith, 1934; Seyffarth, 1940; Denny-Brown, 1949; Gordon & Holbourn, 1949; Bigland & Lippold, 1954; Li, Shy & Wells, 1957). This would seem to imply that the muscle fibres innervated by a single motor nerve fibre are grouped in compact bundles. It is known, however that efferent fibres supply the widely separated end-plates of muscle fibres arranged end to end in the cat tenuissimus (Adrian, 1925) and sartorius muscles (Cooper, 1929). The possibility clearly exists that, in all muscles, fibres of one motor unit may be much dispersed.

Evidence for the manner in which fibres are actually distributed is both scanty and indirect. The scatter of atrophied patches in disease of the motoneurone (Wohlfart, 1949) and after experimental section of spinal roots (Van Harreveld, 1947) suggests that the motor unit does not form a single compact bundle. The spatial spread of potentials recorded by electromyography also points to a substantial overlap of active units (Jasper & Ballem, 1949; Buchthal, Guld & Rosenfalck, 1957). According to the last authors, in the human biceps the motor unit extends over a region with a diameter of about 5 mm. A direct anatomical analysis of the ramifications of a single motor nerve fibre is made exceedingly difficult by the profuse branching and interlacing of fibres in the muscle. Nevertheless, Tergast (1873) and Feindel (1954) have claimed that under special conditions they could follow the branches of single fibres, in cat and rabbit muscles respectively. Their descriptions show clear overlap of motor units; muscle fibres of the same motor unit may be separated by as many as eight unrelated fibres according to Feindel.

The distribution of a motor unit in a muscle can be shown by isolating and then stimulating a single motor nerve fibre and analysing the corresponding muscle action potentials. This approach has not hitherto been attempted with unqualified success (Denslow & Gutensohn, 1950, 1951). By such a method we have found that in the rat diaphragm the muscle fibres activated by one single motor nerve fibre are irregularly scattered over a relatively wide region.

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METHODS

Dissection. Albino rats (Wistar origin) weighing 100–200 g were anaesthetized with ether. The two halves of the diaphragm were removed, together with the phrenic nerves, and left for some minutes in a Ringer–Locke solution aerated with 5% CO_2 in O_2, at room temperature. A single motor nerve fibre was isolated from the phrenic nerve at a point about 2–3 mm away from the corresponding hemidiaphragm and all the remaining fibres were cut. For confirmation, all preparations were examined either at the beginning or the end of an experiment with a microscope (×60). The hemidiaphragm was trimmed so as to preserve only that portion which contained the active motor unit.

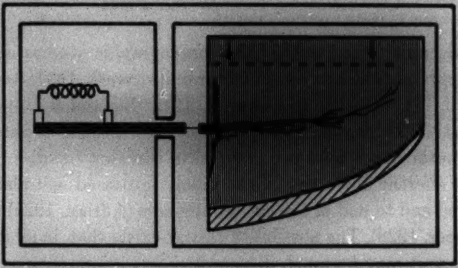

Fig. 1. Diagram showing the recording chamber, with the phrenic nerve on stimulating electrodes on the left, and a portion of the diaphragm on the right. The broken line indicates the approximate path of the surface electrodes which recorded the potentials illustrated in Fig. 2; the two arrows mark the area of distribution of that motor unit.

Recording chamber (Fig. 1). The preparation was fixed in a Perspex chamber with two compartments separated by a sliding partition. On one side the muscle and the distal part of the phrenic nerve, including the isolated fibre, were bathed in Ringer–Locke solution identical with that described by Liley (1956) except for a greater glucose content in some experiments (5 g/l. instead of 2 g/l.). The solution was very effectively stirred by constant vigorous bubbling of the aerating mixture of CO_2 and O_2. It was kept at room temperature (22–24° C), and it was not changed except by the addition of small amounts of fresh solution when, at intervals of some hours, surface froth and scum had to be removed by suction. The proximal part of the phrenic nerve extended through the partition into the adjacent compartment, where it rested on a pair of platinum stimulating electrodes submerged in a pool of liquid paraffin.

Recording technique

The solution in the muscle compartment was earthed through an Ag–AgCl coil in series with a resistance of 50 Ω (across which were applied calibrating and balancing potentials).

A. *External recording.* The electrodes were fine platinum wires (diameter 175 μ) fused in glass, separated by a distance of 0·19 mm. They were connected to the input of an R-C differential preamplifier, and were attached to a micromanipulator (Eccles, Fatt, Landgren & Winsbury, 1954) which made possible a detailed survey of the surface of the muscle. Further amplification was by a conventional R-C amplifier, with a flat frequency response up to about 20,000 c/s.

B. *Intracellular recording.* Glass micropipettes were made in the device described by Winsbury (1954), and then filled with 3 M-KCl by boiling under reduced pressure. They had an electrical resistance of 7–20 MΩ. They were connected by a saline-agar bridge, and then an AgCl–Ag link to the grid of a cathode follower, whose output was fed into an R.C. amplifier and a millivoltmeter.

Stimulation. The stimulator output consisted of condenser discharges (with a time constant of 15 μsec), slightly modified by an isolating transformer. Care was taken to have the intensity of the stimuli always at least three times the threshold intensity.

Analysis of records. The oscilloscope tracings were photographed with a Grass camera, and then analysed after suitable magnification (×8–10).

Histological techniques

Glycogen stain. The diaphragm was fixed in 10% (v/v) formol in ethanol saturated with picric acid, dehydrated in absolute ethanol and embedded in paraffin. The 10 μ sections were floated on 70% ethanol and then covered on the slides with a film of celloidin. The sections were stained in haemalum and Best's Carmine. Contact with water was avoided at all stages. Control sections of rat liver were treated in the same manner, and the specificity of the glycogen reaction shown by preliminary spitting on some of these sections.

Phrenic nerve. The nerve was stretched on a rectangular glass frame and fixed in Fleming's fluid. 10 μ paraffin sections were stained in haemalum and counter-stained in aqueous eosin.

RESULTS

The isolated single-fibre preparation is exceedingly fragile, but once set in the recording chamber it will survive under the conditions already described for a period of time more than adequate for the purposes of the experiment. In two cases, single fibres conducted impulses to the diaphragm for 12 hr; during most of this time the fibre was stimulated at frequencies varying between 2 and 25/sec, and, although at the end of this period there was a diminution in contraction, the isolated fibre had not obviously deteriorated.

The results are based upon eight successful experiments. In four cases the preparations had only one nerve fibre. In the four remaining cases two fibres were actually left for technical reasons: in three of these preparations, there was only one active motor fibre. This was shown by exhaustive tests for differences in threshold, particularly with two stimuli and during a tetanus, since even a minute difference is usually revealed clearly during the recovery period. Close examination with the microscope was also of help in showing injured parts of one fibre. In one case we had two active motor fibres but the two motor units could be distinguished by a small difference in thresholds. The results obtained with these two-fibre preparations are less obviously significant, but they are included since they confirmed in every respect what was found with single-fibre preparations.

Visual inspection

When a single motor nerve fibre is stimulated it is immediately and very strikingly clear to the naked eye that the active muscle fibres are dispersed over a relatively wide area. Instead of a localized contraction, one sees surprisingly diffuse movement, differing from the normal twitch quantitatively rather than qualitatively. Although we have been able to record electrically from only eight preparations, we have seen another five single-fibre preparations which did not survive further manipulation. In no case did we observe sharply circumscribed contractions. The region involved differed in various preparations; the largest was as much as half the total area supplied by the phrenic nerve.

With a dissecting microscope (× 10–25), one can discriminate to some extent between different groups of fibres; yet it is almost impossible to identify single contracting muscle fibres. But if the latter are stimulated tetanically, they can be localized approximately, because adjacent resting fibres are usually drawn into a characteristic zigzag pattern. When a single stimulus to the nerve is near threshold, the visible contraction behaves in a perfectly all-or-none manner. However, with repetitive stimulation at higher frequencies (> 10/sec), the distribution and the amount of contraction fluctuate and diminish substantially.

A. *External recording*

The diaphragm is a thin, flat, but comparatively wide muscle which can be surveyed very conveniently with a pair of electrodes probing over its surface. It is also short enough for its fibres to extend from origin to insertion. Accordingly, Coërs (1953) found only a single, narrow innervation band in the rat diaphragm. In confirmation, while recording with intracellular electrodes from widely separated parts of the same muscle fibre, or when stimulating fibres directly after curarization (Krnjević & Miledi, 1958), we have never seen any indication of a series of fibres arranged end to end.

The amplitude of the signals depended of course on the height of the electrodes above the muscle. This was kept approximately constant in any one series of explorations. Care was always taken to avoid dragging electrodes along the surface.

The electrical potentials recorded near the surface of a hemidiaphragm while stimulating a single nerve fibre (2/sec) are shown in Fig. 2. The electrodes moved across the muscle, roughly parallel with the innervation band, but 2–3 mm away from it. Active fibres were spread over an area of the posterior half of the muscle 8·15 mm wide (see Fig. 1). The sequence of variable peaks, troughs and inactive gaps corresponds to the activity of widely dispersed fibres. External recording in a volume conductor with relatively large electrodes gives compound potentials from fibres which are comparatively close together. Better discrimination was obtained with the two electrodes set in line with, rather than at a right angle to, the fibres. Clearly the shape and amplitude of the potentials recorded by such electrodes is determined by their distance from the active fibres, both in a horizontal and in a vertical plane. Although single potentials are not obvious except where one active fibre is relatively isolated (e.g. at 9·15 mm in Fig. 2) in most cases the basic units can be identified because they may differ in, (*a*) the point at which their maximum amplitude is recorded, (*b*) their latencies, (*c*) their refractory periods, and (*d*) their ability to respond to tetanic stimulation. This laborious counting procedure was only performed in full detail in three cases. The counts were 7, 13 and 17 definite fibres respectively. The fibres were not distributed according to any obvious plan, as can be seen in Fig. 3, which shows diagrammatically the relative position of the active fibres in the muscle irrespective of depth. The

Fig. 2. Distribution of potentials evoked by the activity of a motor unit. Single nerve fibre preparation, stimulated at 2/sec. The records were obtained by displacing a pair of electrodes across the surface of the muscle in a direction perpendicular to that of the muscle fibres and parallel to the branch of the phrenic nerve (about 3 mm distant) that innervates this part of the muscle. The electrodes were oriented in the same direction as the muscle fibres. The numbers beneath each record indicate the distance of the electrodes (mm) from the level at which the phrenic nerve enters the muscle.

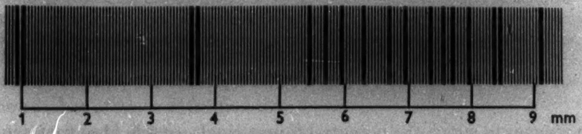

Fig. 3. Schematic representation of the distribution irrespective of depth of muscle fibres in a motor unit. Diagram constructed from data partly shown in Fig. 2. The columns represent fibres of 50μ average diameter. The fibres of the motor unit are illustrated by the solid columns. They are placed according to the position at which the potential of each fibre reached its maximum. Individual fibres were identified by their latency and refractory period.

vertical lines represent very approximately muscle fibres, assuming that their average diameter is 50μ. In fact, the actual density of fibres would have been even greater; we found in histological sections that most fibre diameters were between 25 and 50μ, in agreement with Muscholl (1957).

Fig. 4. Potentials recorded at different distances from the muscle fibre shown in Fig. 2 at 9·15 mm. The pair of differential electrodes was oriented in line with the direction of the muscle fibre and moved perpendicular to it. The figure in each record denotes the distance of the electrodes measured in mm from the point at which the potential is maximal. The record at the extreme right shows some complication due to the potential contributed by other fibres of the unit.

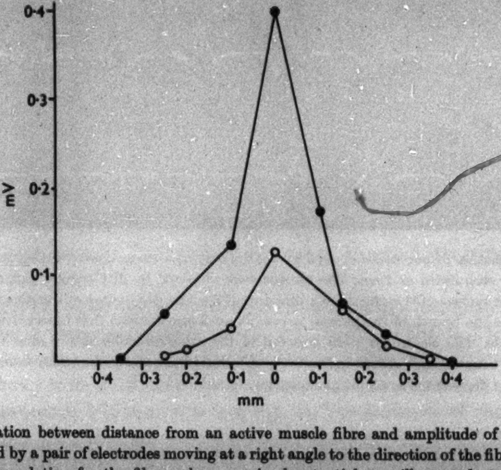

Fig. 5. Relation between distance from an active muscle fibre and amplitude of the potential recorded by a pair of electrodes moving at a right angle to the direction of the fibre. ● and ○ show the relation for the fibres whose maximal potentials are illustrated at 6·75 mm and 9·15 mm respectively in Fig. 2. The point at which the amplitude is maximal is taken as zero distance: i.e. no allowance has been made for the depths at which the fibres were located.

The manner in which the potential from a single muscle fibre varies in shape and amplitude as the differential electrodes approach and then recede, along the surface, is shown in detail in Figs. 4 and 5. Similar changes were seen in an experiment in which the electrodes were moved in a vertical plane, in line with the active fibre. The depths of the active fibres were not measured exactly, but they were known to within about 0·1 mm. It is clear that with this method of recording the amplitude of the potential from a single fibre is reduced to 10μV or less within a distance of about 0·5 mm.

B. *Intracellular recording*

Intracellular recording has, of course, the great advantage of identifying single active fibres with certainty. However, it is by no means convenient for mapping out the total distribution of a motor unit. The individual fibres are scattered over a volume of muscle which is very large compared to that of one fibre. Localization of contracting areas by inspection gives only a very rough indication; the process of searching for an active fibre with fine micro-electrodes is somewhat like looking for a needle in a haystack.

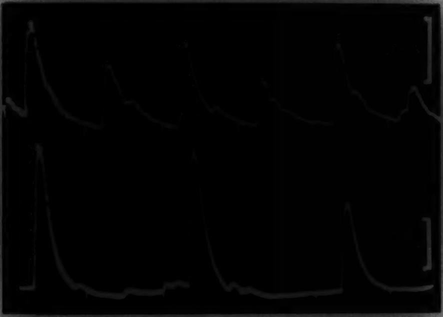

Fig. 6. Intracellular potentials recorded simultaneously from two end-plates of a motor unit. Single nerve fibre preparation stimulated at 64/sec. End-plate potentials are seen superimposed on a background of spontaneous miniature potentials. The distance between the electrodes was approximately 3 mm.

Several conclusions based upon the observations with surface electrodes were confirmed with intracellular electrodes recording from fibres belonging to single motor units.

(*a*) Single active fibres are found over a wide region of muscle.

(*b*) Active fibres may be quite superficial or more or less deep within the diaphragm. It is possible to insert an electrode into fibres showing the characteristic action potential in the 1st and the 8th layer from the surface.

(*c*) The number of fibres identified with intracellular electrodes varied between five and ten in different motor units.

The wide separation between two active end-plates can be shown directly with two micro-electrodes about 3 mm apart (Fig. 6). We have not seen activity in contiguous fibres; the electrode always passed through several silent fibres before it reached another active one.

C. *Behaviour of fibres belonging to a motor unit*

It became clear at an early stage in the course of these experiments that the activity of individual members of the motor unit had certain distinguishing features. We observed definite differences in the latency of arrival of the action potential, in the refractory period, and in the ability of different fibres to respond to repetitive stimulation.

(*a*) Differences in latency are obvious in the records of Fig. 2. The interval between the stimulus artifact and the apex of the potential was measured for eleven separate fibres in one motor unit; the range of values was 5·2–7·6 msec. Only in two cases were the latencies identical. There was a general but by no means regular tendency for the intervals to increase as the electrode moved from the proximal towards the distal edge of the mucle (in a direction roughly parallel with the corresponding intramuscular branch of the phrenic nerve, as shown in Fig. 1).

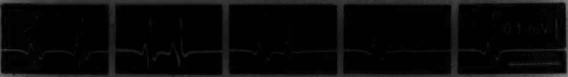

Fig. 7. Effect of decreasing interval between two stimuli on the responses recorded at 7·3 mm in the motor unit shown in Fig. 2. The pairs of stimuli were applied at 2/sec. It can be seen that the four fibres contributing to the potential at this point reach their refractory period at different intervals.

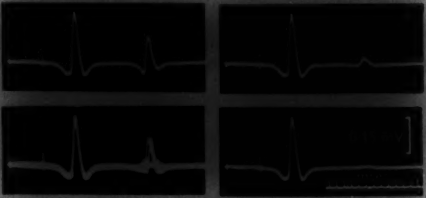

Fig. 8. Responses obtained during the refractory period at 3·7 mm in the motor unit illustrated in Fig. 2. Pairs of stimuli applied at 2/sec, with a constant interval of 4·5 msec. Although the refractory period for neuromuscular propagation is similar in the two muscle fibres that make up the potential recorded from this point, independent small fluctuations cause the fibres to fail asynchronously. Each record is composed of 1–5 superimposed traces.

(*b*) In the wake of a first impulse, differences in the duration of the refractory period for neuromuscular propagation cause certain fibres to become excitable before others (Fig. 7). The refractory period of nine fibres was measured by applying pairs of stimuli at a frequency of 2/sec. The durations ranged from 3·1 to 4·6 msec. Only two pairs of fibres gave identical values. However, even where the refractory periods are similar, individual fluctuations are independent; this causes potentials of the different fibres to fail asynchronously (Fig. 8).

(c) Sooner or later all muscle fibres begin to fail to respond when tetanic stimulation (direct or indirect) is above a certain frequency (Krnjević & Miledi, 1958). The time and frequency at which failure occurs, and also the mode of failure, is apparently characteristic of each fibre and not of the motor unit as a whole. This can be shown with extracellular or intracellular electrodes. In Fig. 9 recognizable single components of the externally recorded compound potential are beginning to fail in an all-or-none manner while others maintain their activity. In Fig. 6 end-plate potentials from two separate fibres are seen simultaneously with two intracellular electrodes. Intermittent failure of propagation occurs in only one fibre. More or less regular variations in the amplitude of end-plate potentials are very commonly seen during tetanic stimulation (see also Krnjević & Miledi, 1958).

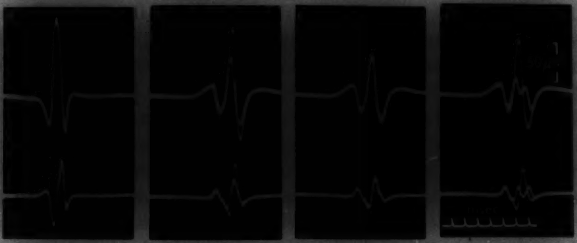

Fig. 9. Asynchronous failure of activation of muscle fibres in a motor unit caused by tetanic stimulation of the nerve fibre at 20/sec. 45 sec after beginning the stimulation the potential (*A*) is similar to the one recorded before the tetanus. 30 sec later (*B–D*) the fibres show intermittent failure. Each record is composed of two to three superimposed traces. The lower traces are the electrically differentiated records of the potential shown in the upper traces.

Histological investigations

Identification of a motor unit. An attempt was made to obtain a permanent record of the distribution of the active fibres in a single motor unit preparation by staining for glycogen in histological sections. However, there was too little glycogen in the muscles to allow one to distinguish between fibres which had and those which had not been contracting.

Certain measurements were made in these sections with the following results relevant to the present study:

Thickness of the diaphragm, 0·31–0·78 mm, with an average of 0·5 mm.
Fibre layers in the diaphragm, 12–24, with an average of about 17.
Diameter of fibres, mostly between 25 and 50μ; the largest diameter observed was 80μ.

Innervation ratio. The total number of fibres counted in microscopic sections of the rat phrenic nerve was about 400. From a width of some 25 mm we estimated that the total number of muscle fibres in the rat diaphragm was about 10,000. The innervation ratio would therefore be 25:1. This is only a very approximate ratio, which makes no allowance for the presence of sensory fibres in the phrenic nerve. However, these are not likely to exceed 10% of the total, well within the margin of error of the estimate of fibres in the diaphragm (probably ±20%).

DISCUSSION

Evidence has been presented that in the rat diaphragm each motor unit extends over a relatively large area of muscle. The reason for this is not a high innervation ratio but a wide scatter of individual fibres. The three counts of fibres were actually somewhat lower than might be expected. Even when allowance is made for afferent nerve fibres, innervation ratios of muscles, other than those controlling movements of the eyes and the face, are usually 100 or more (Clark, 1931; Feinstein, Lindegard, Nyman & Wohlfart, 1955). In the rat diaphragm, however, the over-all ratio is apparently only about 25. We do not think that the numbers we have counted are gross underestimates of the active fibres, for the following reasons:

(1) The muscles had an average thickness of 0·5 mm, as determined in histological sections (Creese, 1954, gives an average of 0·6 mm in 150–200 g rats). Judging by the change in amplitude of potentials from single fibres with increasing distance (Fig. 4), the surface electrodes should detect all active fibres, except possibly those from the very deepest layers. (2) Even when deep potentials are masked by overlying activity, it is unlikely that they cannot be revealed by close inspection during tetanic stimulation, or when pairs of stimuli are employed. Some active fibres perhaps do escape detection, but their number would probably not change our counts very substantially. A more serious cause of error in estimating the size of the motor unit might be failure of propagation in a substantial fraction of the fibres. Partial block of transmission in isolated preparations is well known (Adrian & Lucas, 1912; Brown & Harvey, 1938) and we have observed the absence of activity in some fibres, right at the beginning of an experiment, both with the rat diaphragm *in vivo* and gracilis *in situ*. However, when the rat diaphragm is kept at room temperature, less than 5% of the fibres fail to respond to nerve stimulation at 2/sec even after a period of 3 hr (Miledi, unpublished observation). Muscholl (1957) found action potentials in 252 out of 254 impaled fibres in the rat diaphragm. This source of error can never be totally discounted, but it can be very much reduced by having a fresh preparation at room temperature, with plenty of O_2, glucose, etc. In order to minimize the effects of injury we made an initial count of the active fibres with recording by surface electrodes.

Several of the observations lead to conclusions which are of some relevance to the interpretation of electromyography in general. (1) It should be recognized that the term 'motor unit', as commonly used in electromyography, does not correspond to Sherrington's original description (Sherrington, 1925). Potentials should not be ascribed to entire motor units without direct evidence that the whole unit is really compact. In a recent paper, Buchthal *et al.* (1956) described very sharp electrical 'units' recorded with micro-electrodes in the human biceps, which seem to be very much like spikes from single fibres. The authors' conclusion that they are probably fromgroups of, on the average, nineteen fibres is based upon rather indirect considerations. It seems more likely that the spikes do represent not more than one or two fibres, and that the authors have either underestimated the volume of distribution of the motor units, or overestimated the appropriate innervation ratio (or both).

(2) Although the motor unit is a functional unit, it cannot be assumed that the individual muscle fibres of which it is made up all behave in exactly the same manner and have the same characteristics. There may be substantial differences in latency and in refractory period, and in the respective rates of discharge at different frequencies of stimulation. It is possible to record several millimetres apart potentials quite dissimilar in amplitude, shape, latency and refractory period, and even firing at different frequencies, yet both belong to the same motor unit. In fact, if a muscle were stimulated artificially through its nerve, one could not ascribe definitely two such potentials to the same motor unit. In voluntary contractions, however, motoneurones are not likely to fire exactly synchronously. Even if two muscle fibres which belong to the same motor unit have distinct rates of discharge (Figs. 6 and 9), their interconnexion ought to become evident upon close examination of the relative timing of their respective impulses.

According to Lorente de Nó (1947) a simple relationship cannot be expected between the amplitude of a nerve potential recorded in a volume conductor and the distance from the active source. However, Buchthal *et al.* (1956) have claimed that the amplitude is a simple logarithmic function of the distance. The logarithmic index which they calculate from their electromyographic data depends very much upon the assumed least distance between their multiple electrode and the active fibre. Since the exact distance was also not determined in our experiments, it did not seem profitable to derive, using an arbitrarily assumed zero distance, what is in any case only an approximate index. It is worthy of note that the signal recorded from an active muscle fibre with two differential electrodes diminishes to 10μV or less within a distance of about 0·5 mm.

The wide scatter and apparent irregularity of innervation by a single motor nerve fibre raise some interesting questions in relation to the manner in which the connexions between nerve and muscle originally take place. Weiss (1955, p. 360) suggested that the repeated peripheral branching of motor fibres occurs after, and as a result of, the consecutive divisions of young muscle fibres. With such a scheme, the most likely result would seem to be motor units in compact bundles. Wide scatter of innervation, on the other hand, would occur if effective collateral branching became possible as soon as further progress ceased at the growing tip, i.e. when contact was established with a muscle fibre. This hypothesis is in keeping with the general belief (Weiss, 1955) that the rate of axonal growth and branching is limited by the available flow of neuroplasm from the cell body.

SUMMARY

1. The distribution of single motor units in the rat phrenic-hemidiaphragm preparation has been studied by isolating and stimulating single motor nerve fibres and recording the electrical potentials of active muscle fibres with extracellular and intracellular electrodes.

2. The potentials were scattered irregularly over areas several millimetres in width, up to nearly half the total area of muscle available. They were not confined to any particular depth within the muscle. There was considerable overlap of motor units.

3. In those motor units in which detailed counts were made, the numbers of fibres which could be distinguished with certainty were 7, 13 and 17 respectively.

4. Potentials recorded from active muscle fibres belonging to the same motor unit may differ in latency, refractory period and even frequency of discharge; with extracellular recording, there may also be large differences in shape and amplitude.

5. The signal recorded from a single active muscle fibre with differential electrodes is reduced to $10\mu V$ or less within a distance of about 0·5 mm.

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FAILURE OF NEUROMUSCULAR PROPAGATION IN RATS

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Many interpretations have been given of the mechanism of muscle fatigue. It is evident that deficient contraction may result from failure at any point in a long chain of events. Various authors disagree as to which link in this chain is the most vulnerable. However, all agree that the nerve trunk is for practical purposes indefatigable, and most authors recognize the occurrence of two main types of peripheral fatigue: fatigue of neuromuscular transmission, and fatigue of the contractile system (del Pozo, 1942; Steiman, 1943; Rosenblueth, 1950; Naess & Storm-Mathisen, 1955). Failure of neuromuscular transmission is commonly ascribed to a deficiency or an excess of transmitter substance (Feng, 1937, 1941; Rosenblueth & Morison, 1937). Other schools of thought may be distinguished. Merton (1954) places all emphasis on fatigue of the contractile mechanism, the occurrence of which, on the other hand, is denied by Ramsey & Street (1941, 1942). Other authors have stressed the importance of fatigue in the central nervous system as the determining factors in fatigue of voluntary contraction (Waller, 1891; Reid, 1929).

In the experiments about to be described, we have studied with intracellular micro-electrodes both *in vitro* and *in situ* the mechanism of neuromuscular failure in rats; a number of observations were made which, we believe, may throw some new light on this subject. A preliminary account of some of these experiments has already appeared (Krnjević & Miledi, 1957).

METHODS

All experiments were performed with muscles from albino rats of Wistar origin, weighing between 100 and 250 g.

Experiments in vitro

The rat phrenic-diaphragm preparation was obtained as described by Krnjević & Miledi (1957). A number of technical changes were made in the course of time which tended to improve the condition of the isolated preparation and to simplify the experimental procedures. Initially the muscle chamber was irrigated with aerated Ringer–Locke solution (Liley, 1956*a*), but it was found both more effective and more convenient to bubble the gas (5% CO_2 in O_2) directly into the chamber. There is good reason to believe that the muscle is preserved in a better condition if the Ringer–Locke solution is not changed at frequent intervals (Zierler, 1956). The preparation was kept either at 37° C or at room temperature (22–24° C); we usually preferred the latter as we have regularly observed a rapid decline in the contraction of the muscle and the response of the nerve after some 6–8 hr at 37° C. At room temperature, by contrast, the preparation continued to function adequately for 12 hr or more (cf. Table 1).

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* Fellow of the Rockefeller Foundation.

The phrenic nerve was passed through a partition into an adjacent chamber filled with paraffin oil. There it rested on a pair of platinum stimulating electrodes, and, in many experiments, on an additional pair of platinum recording electrodes which monitored the nerve impulse as it travelled to the diaphragm.

Electrical recording techniques

Intracellular recording in single muscle fibres. Glass micropipettes were drawn out (Winsbury, 1954) and filled with 3 M-KCl. They had a short connexion to the grid of a cathode follower mounted on a rigid micromanipulator (Eccles, Fatt, Landgren & Winsbury, 1954). The electrodes had a resistance of between 7 and 30 MΩ. The output of the cathode follower was amplified in a conventional manner, and also fed directly into a millivoltmeter. Calibrating signals were applied across a 50Ω resistance between earth and the muscle chamber.

The electrodes were inserted vertically under direct vision, with the help of a dissecting microscope (× 10–25). When end-plate activity was studied, the electrode was aimed into the region around the terminations of fine intramuscular nerve branches, which were easily made visible by transillumination. In the great majority of cases spontaneous miniature end-plate potentials (Liley, 1956*a*) were recorded at the first trial. The end-plates were apparently grouped in these regions, which correspond to the innervation band described by Coërs (1953); further insertions into other fibres would usually also reveal spontaneous potentials, firing at distinctive rates.

Simultaneous intracellular recording with two micro-electrodes. The technique was similar to the above, except that the electrodes had to be inserted at an angle of about 20° to the normal because of interference between micromanipulators.

Recording from nerve trunk. The electrode nearer the stimulating electrode was connected to the earth of the muscle chamber. The conduction distance was of the order of 1 cm.

Experiments in situ

The rats were anaesthetized with intraperitoneal pentobarbitone sodium (Nembutal, Abbott Laboratories, 5 mg/100 g at intervals of about 1 hr) and fixed in a supine position on a cork board. The skin of the ventral aspect of the left thigh was reflected to display the underlying muscles. The nerve to gracilis anticus was freed along the upper border of that muscle, cut, and then mounted on platinum stimulating electrodes. Evaporation from the muscle and the nerve was prevented by filling the space above and between the muscles with liquid paraffin. The method by which potentials in the gracilis were recorded with intracellular electrodes was in all other respects similar to what has already been described for the diaphragm.

Gracilis anticus is a much thicker muscle than the diaphragm; it cannot be transilluminated conveniently, especially *in situ*. For that reason, we did not find it possible to trace fine nerve branches to their endings *in situ* (cf. Bryant, 1957) and thus to localize regions rich in end-plates. However, the intramuscular course of the motor nerve can be seen quite clearly in muscles stained with methylene blue and cleared in glycerol. The principal nerve runs along the whole length of the muscle, approximately in the middle of its deep surface, giving off fine lateral branches at most levels. The most profuse branching, however, is at the distal end of the muscle, and also near and above the nerve entrance, at the pelvic end. We found accordingly that end-plate potentials (e.p.p.'s) were recorded most easily in those regions. In practice, end-plates were located by the presence of spontaneous miniature potentials (m.e.p.p.'s). As in the diaphragm, end-plates tended to be grouped, albeit at different depths.

Stimulation

Indirect. In most experiments, the muscles (*in situ* and *in vitro*) were stimulated indirectly. The stimuli were essentially condenser discharges, somewhat altered by passage through an isolating transformer. Prolonged tetanic stimulation was possible up to a frequency of about 600/sec. During an experiment several checks were made on the nerve threshold, and the actual stimuli used were always well above maximal.

Direct. In order to test the threshold of muscle fibres to direct stimuli, bipolar stimulating electrodes were placed on the surface of the muscle. At first they were fine platinum wires (0·18 mm) fixed in glass capillaries. Suprathreshold stimulation of even a few muscle fibres required the application of high voltages and long pulses. With tetanic stimulation this soon gave rise to substantial polarization of the electrodes, which falsified threshold measurements. Satisfactory experiments resulted when non-polarizable electrodes were utilized: these consisted of twin glass capillaries (diameter 0·9 mm) about 1 mm apart, filled with an agar-Ringer solution, with a Ag-AgCl junction.

Direct application of ACh

The method was similar to that described by Nastuk (1953) and del Castillo & Katz (1955). ACh was delivered electrophoretically in quick jets by passing 0·1–1·0 msec square outward pulses through glass micro-electrodes containing a 2–4 M solution (acetylcholine chloride, Roche Products). A variable d.c. bias was also applied to the electrodes to control spontaneous diffusion. ACh-potentials were produced with a current equivalent to a total charge less than 5×10^{-12} C, that is, less than $1{\cdot}5 \times 10^{-17}$ equivalent of ACh if we assume the transference number to be 0·3 (del Castillo & Katz, 1955).

Estimations of muscle electrolytes

The muscles were weighed and then dried during the night at 105° C. The dry tissue was soaked in distilled water for 48 hr: the resultant Cl solution was later titrated potentiometrically with standard $AgNO_3$ by a differential method with an error of about 1 %. The tissue was then ashed, either in a muffle furnace at 500° C, or by digestion with conc. HNO_3 at 80° C. After solution and/or suitable dilution of the ash, the Na and K contents were estimated with an Evans Electroselenium flame photometer. The mean recoveries of Na and K in a series of nine control experiments were 103·4 % (S.D. ±1·7 %) and 102·8 % (S.D. ±2·1) respectively.

RESULTS

If a micro-electrode is inserted into a contracting fibre (not near an end-plate) in a muscle stimulated indirectly at a frequency not very much above 10/sec, it is often possible to record quite satisfactorily, in addition to a resting potential, a regular sequence of action potentials (Fig. 1). It may be noted that the resting membrane potential of quiescent muscle fibres was usually of the order of 75 mV, as found by Lundberg & Quilisch (1953), Liley (1956*a*) and Li, Shy & Wells (1957). However, values were often lower in fibres which were contracting, especially after a substantial period of recording. This was to be expected since movement would tend to prevent perfect sealing of the membrane around the electrode. The changes which are described cannot have been caused by depolarization of the muscle fibres because they can be observed with extracellular electrodes (except for failure of propagation along the fibre); presynaptic events and the threshold at a point several millimetres away from the recording electrode would not, in any case, be affected by variations of the resting potential, while changes in e.p.p.'s were only considered significant during periods of very stable resting potential. These action potentials are clearly all-or-nothing in nature, and their frequency of occurrence corresponds to that of the stimuli. This will be true for a long period (certainly up to 10–15 min) if the preparation is well supplied with O_2.

If the frequency of stimulation is now increased suddenly to about 40/sec, there is an immediate corresponding increase in the frequency of action potentials, which, for a short time, still bear a one-to-one relation to the stimuli. However, after a variable period an intermittent failure of propagation is

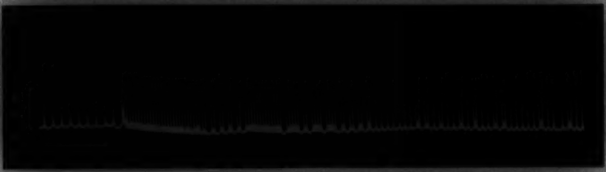

Fig. 1. Rat diaphragm at room temperature. Records obtained from a single muscle fibre with an intracellular electrode at a point 3–4 mm away from the end plate. The nerve was stimulated at 14/sec for several minutes and then the frequency was changed to 40/sec. The intensity of stimulation was about ten times threshold. Resting potential 49 mV. The first failure occurs 0·75 sec after the onset of the 40/sec stimulation. Increasing the strength of the stimuli did not prevent the failures.

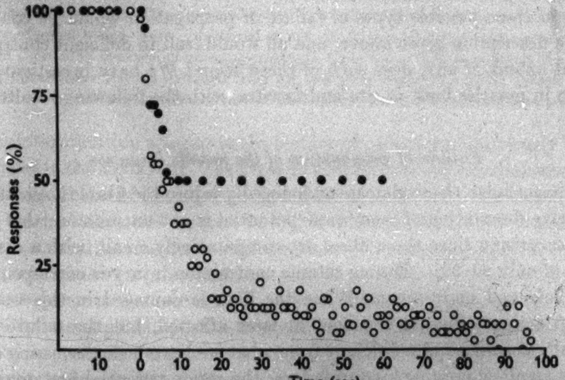

Fig. 2. Failure of neuromuscular propagation as recorded in two muscle fibres in the experiment partly illustrated in Fig. 1. Ordinates: number of responses as percentage of the number of stimuli per second. At time 0 the frequency was changed from 14/sec to 40/sec. ○, responses illustrated in Fig. 1. The activity of the other fibre, ●, was not photographed beyond 60 sec; observation of the oscilloscope showed that propagation failed completely 2 min 45 sec after the start of the 40/sec stimulation.

usually seen, which is rapidly progressive in character, and may lead to a condition of more or less complete block (Figs. 1 and 2). The final steady state may not occur for several minutes, and the degree of block may vary considerably with different fibres and different rates of stimulation (Fig. 2). In a good preparation these changes are apparently fully reversible with rest, but the initial condition may not return for some appreciable time.

This sequence of events immediately prompts the question: Where does the failure of propagation take place? Clearly, failure could occur at any one of several sites. There might be:

(1) failure of propagation of the impulse along the muscle fibre, possibly because of local damage caused by the micro-electrode;
(2) failure of initiation of a conducted response in the muscle fibre by the localized e.p.p.; this might occur because the e.p.p. has become substantially smaller than normal and/or because of a raised threshold of the muscle fibre;
(3) failure of neuromuscular transmission, either because of complete exhaustion of ACh or inactivation of its liberating mechanism;
(4) presynaptic failure of propagation, which, in an undamaged nerve, would be most likely to involve the fine nerve terminals or sites of branching; or
(5) failure of initiation of a conducted nerve impulse because of a rise in threshold at the point of stimulation.

Each of these possible types of failure of propagation would be consistent with the description given above, and all would lead to deficient contraction. To what extent, if any, does each of these occur? We have investigated this problem in muscles both *in situ* and *in vitro*, with the following results.

Failure of propagation of the muscle impulses

An intracellular electrode can undoubtedly injure the fibre. Even at rest a progressive diminution of membrane potential is not uncommon; this should not be surprising since these fibres are comparatively small, with a diameter usually of only 20–50μ. During tetanic contraction injury is correspondingly more likely and more severe. When the fibre is damaged in this way, the recorded action potential is sooner or later affected, but the failure which eventually occurs is quite different from what we have been discussing above. It is not an all-or-nothing kind of failure: the action potential first diminishes somewhat and its duration increases. A notch then appears which becomes more and more prominent, and tends to occur later, until two distinct but smaller potentials are visible (Fig. 3). The second later vanishes abruptly, whereupon one is left with a 'broken' potential, which may remain without much change, or may diminish more or less rapidly without further breaks.

Similar broken potentials caused by micro-electrodes have been observed in rat muscle by Li *et al.* (1957) and in frog nerve by Woodbury (1952).

We have interpreted these observations as follows: the appearance of a notch in the action potential reflects progressively slower conduction of the impulse in the injured region; when the second part of the potential disappears, propagation past the electrode has ceased. The further diminution of the remaining potential occurs because there is a continued decrease of the action potential and also because the impulse stops at an increasing distance from the electrode, which ultimately records only an electrotonically conducted potential.

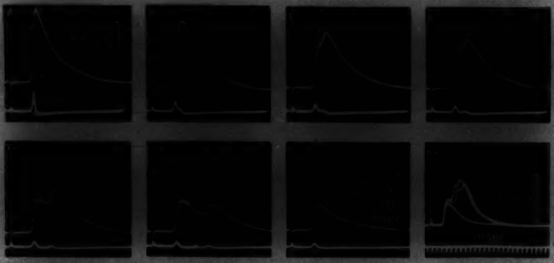

Fig. 3. Diaphragm at room temperature. Upper traces, muscle fibre action potential obtained with an intracellular micro-electrode at a point 3–4 mm away from end-plate during nerve stimulation at 10/sec. The lower traces were obtained simultaneously by electrical differentiation of upper traces. Resting potential 46 mV. *A*, 12 min after beginning nerve stimulation and 1 min after insertion of micro-electrode; *B*, 2 min later; *C–G*, at intervals of about 3 sec. *H*, intracellular records obtained from the end-plate region of another muscle fibre; the potential broke into three components. Each record shows 1–3 superimposed potentials.

This interpretation was put to the test by recording simultaneously with two electrodes in the same fibre. The greatest obstacle to such simultaneous recording is the problem of identifying a given fibre. A convenient solution is based upon the fact that the manner in which a muscle fibre fails intermittently during maximal tetanic nerve stimulation is quite characteristic for that fibre, whether the failure is presynaptic or not (see below). A tetanic stimulus was therefore given to the nerve, and a muscle fibre which was only firing intermittently was impaled with the first electrode. The second electrode was then inserted into muscle fibres in a region proximal to the first electrode. When the insertion was made into a fibre with the characteristic intermittent rate of firing, it was possible to relate the progressive changes at the proximal electrode to the appearance or non-appearance of a propagated potential at the distal electrode. In the most clear-cut experiments, we found that the distal potential did vanish simultaneously with the second component of the breaking potential recorded more proximally (Fig. 4). This type of failure is essentially unphysiological. However, its possibility cannot be altogether ignored where muscle fibres are not entirely homogeneous; e.g. because of multiple innervation or uneven blood supply.

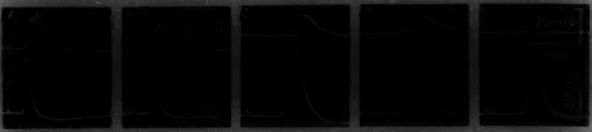

Fig. 4. Failure of conduction of a muscle fibre action potential in a diaphragm stimulated through its nerve at 10/sec. An electrode was first inserted in a distal portion of the muscle fibre, then a second electrode was inserted in a more proximal portion, not near the end-plate. The potentials recorded simultaneously with the distal and the proximal electrode are shown in the upper and lower traces respectively. *A* and *B* illustrate the breaking of the distal potential; *C–E* are progressive stages during the breaking of the proximal potential, which shows a slight notch in *C* and *D* and finally breaks in *E*. Correspondingly, in *E* only a small electrotonic potential is recorded by the distal electrode.

Failure of initiation of a conducted impulse in the muscle fibre

It is quite easy to demonstrate e.p.p.'s in fatigued muscle with intracellular electrodes, but one cannot exclude the possibility that failure of propagation is again due to injury. Potentials recorded intracellularly at end-plates often 'break' so that *two* notches appear. Subsequently, the two later components vanish, leaving a presumably localized e.p.p. (Fig. 3, *H*). We believe that this behaviour parallels that of the damaged muscle fibre already described and that the triple system can be ascribed to the fact that impulses propagate from the end-plate in both directions along the muscle fibre. It is impossible to say whether this type of break is primarily caused or merely precipitated by local damage. Evidence is given below that conducted impulses may fail to be initiated at the end-plate under physiological conditions. It seems to us probable that double notching would tend to occur just before complete failure, since the end-plate region is hardly likely to be perfectly symmetrical.

It can be shown indirectly, but nevertheless unequivocally, that the e.p.p. can fail to initiate propagated responses in the muscle fibre during repetitive stimulation, even in the absence of local injury. This was done quantitatively by comparing the proportion of active muscle fibres (i.e. those responding to every stimulus) with the proportion of active end-plates, in a comparable number of random insertions (approximately 20) in a given time (5 min); the whole procedure was repeated after 5 min rest at a different rate of stimulation. End-plates were always identified by the presence of m.e.p.p.'s. The results of such an experiment on a diaphragm are shown in Fig. 5. The wide divergence of the two curves at all frequencies between 10 and 125/sec shows clearly the existence of a functional discontinuity between the end-plate region and the remainder of the muscle fibre. With experiments *in situ* the results are very similar. One hundred per cent propagation is seen in very few muscle fibres at frequencies of stimulation of 50/sec or greater.

We must now consider the causes of failure of propagation from the end-plate region.

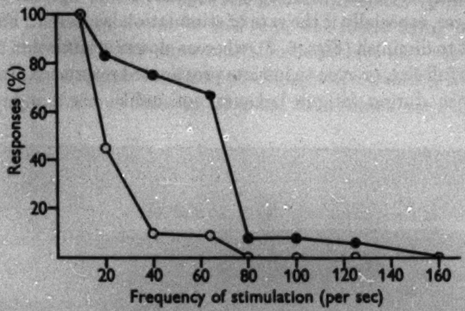

Fig. 5. Proportion of muscle fibres responding (with an e.p.p. or spike) without failure to stimulation of the phrenic nerve for 5 min at different frequencies, when recording intracellularly near end-plates, ●, and away from end-plates, ○, Ordinates, percentage of muscle fibres responding to every stimulus.

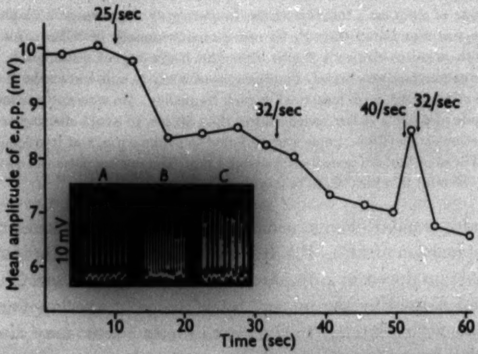

Fig. 6. Diaphragm at room temperature. Decrease in mean amplitude of e.p.p. during continuous tetanic stimulation of the phrenic nerve. About 2 min after beginning the stimulation (16/sec) a micro-electrode was inserted into an end-plate region and e.p.p.'s were recorded continuously. The frequency was changed as indicated at the times marked by the arrows. Resting potential 54 mV throughout the period of observation. Note the recovery of amplitude when presynaptic intermittent failure supervened during stimulation at 40/sec. Inset: Examples of e.p.p.'s from which the above curve was derived; *A* at 1 sec, *B* at 46 sec, *C* at 51 sec (40/sec).

Reduction in amplitude of e.p.p. As already pointed out, if with an intracellular electrode one records the activity of a muscle fibre at or near its end-plate during tetanic stimulation, one sees, sooner or later, a double break of the potential. Ultimately only a non-propagated e.p.p. is left which characteristically varies in amplitude (Figs. 6, 11). These variations may be quite substantial and may occur in a regular sequence. The e.p.p. is susceptible of further changes, especially if the rate of stimulation is altered. Faster stimulation causes it to diminish (Figs. 6, 7), whereas slower stimulation may enable it to recover its full size, or even to initiate propagated responses. In this respect, the e.p.p. seen during fatigue behaves not unlike the e.p.p. of curarized preparations.

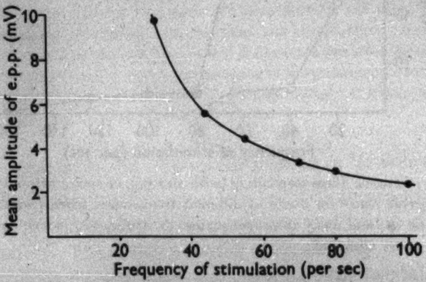

Fig. 7. Amplitude of e.p.p. as a function of the frequency of responses in a diaphragm at 36° C. The muscle was stimulated through its nerve at a frequency of 30/sec. An end-plate was impaled with a micro-electrode 5 min after the beginning of stimulation, and then the frequency was progressively raised. Photographs of 20 e.p.p.'s, taken at about 1 min after the start of the stimulation, were measured at each frequency. No attempt was made to measure the amplitude of e.p.p.'s at frequencies lower than 30/sec, to avoid eliciting action potentials and contractions in the fibre. Some stimuli failed to evoke e.p.p.'s at frequencies higher than 100/sec and even at that frequency after about 1 min. The resting potential was 55 mV during the period (15 min) in which these measurements were taken.

A remarkably similar change occurs with potentials evoked artificially by the direct application of ACh. Fig. 8 shows the progressive diminution of such ACh potentials produced in a diaphragm at the rate of 40/sec. These changes are clearly not caused by the mutual interference of overlapping potentials: ACh potentials with a latency to the peak of only 5 msec may diminish when stimulated at a frequency of even 1/sec.

Increase of the muscle fibre threshold. The threshold of single fibres was measured at various frequencies of direct stimulation, by recording the fibre action potential (in a region free of end-plates) with an intracellular electrode. It was necessary to curarize the preparations both *in vitro* and *in situ* as the intramuscular nerve branches had a much lower threshold than the muscle fibres. For experiments *in situ* the anaesthetized rats of course also needed artificial respiration. The paralysing agent was tubocurarine chloride (Burroughs Wellcome) in a final concentration of 10^{-6} *in vitro* and *in situ*.

The experiments were performed as follows. The stimulating electrodes were lowered until a small part of the muscle was activated by single, comparatively weak, stimuli. Tetanic stimulation was then begun at a certain frequency, with the intensity of the stimuli somewhat above threshold. Fibres were then impaled with the micro-electrode until one was found firing regularly. The threshold of that fibre was determined immediately, and then at frequent intervals. In this manner we observed changes of threshold with time, to a maximum value of about three to six times the initial threshold.

Fig. 8. Potentials produced in a rat diaphragm by the electrophoretic application of ACh in 1 msec pulses ($3{\cdot}5 \times 10^{-8}$ A). Top row: at the left is a single control potential as seen with a high sweep velocity; at the right is the beginning of a series of potentials at a frequency of 40/sec. Bottom row: the last part of the same series 31 sec after the end of the top row; at the extreme right, a single ACh-potential 1·5 sec later gives an indication of the rate of recovery. m.e.p.p.'s are visible throughout. Irregularities in the base line were caused by slight spontaneous twitches. All potentials were obtained by d.c. amplification; the initial level of the base line is indicated by a short line at the extreme right of the top row, and at the extreme left and right of the bottom row.

Up to a frequency of about 10/sec there is very little increase in threshold, even after 10 min or more. At higher frequencies the threshold tends to increase more or less rapidly with time. There is a great deal of variation between fibres in this respect (Fig. 9). In some the rate of change is quite dramatic, so that it is soon impossible to follow it, while in others the onset may be delayed. Despite the substantial variety of behaviour, we did not encounter a fibre capable of responding for more than 2–3 min to stimuli at a rate much greater than about 50/sec, either *in vitro* or *in situ*. Muscle fibres *in situ* were rather more resistant to the effects of tetanic excitation than fibres *in vitro*.

One or two other observations may be of interest. When the threshold of a fibre begins to overtake the intensity of the stimulus, a period of intermittent failure was observed. In most cases it was quite short, and was followed by total irresponsiveness. The change in excitability was not immediately reversible, and even if the tetanic stimulation was suspended the return to normal was quite slow, albeit ultimately complete. It was only possible in one case to remain in the same fibre long enough to observe the gradual recovery of excitability: the initial threshold was reached in 8 min. This was in keeping with visual observations of the time needed for contractions to become apparent (about 15 min). Very similar results were obtained when the activity of single muscle fibres was recorded with external electrodes to exclude any effects of injury (Fig. 10).

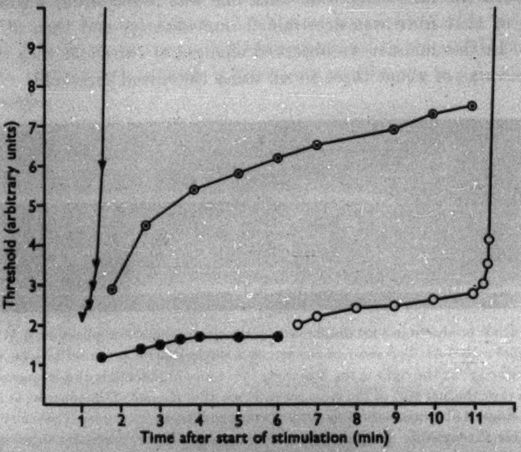

Fig. 9. Curarized diaphragm at room temperature. Threshold of four muscle fibres after the start of direct stimulation at different frequencies. ● and ○, two different fibres observed during the same period of stimulation at 20/sec; ▼, at 32/sec; ⊙, at 40/sec.

Complete exhaustion of the transmitter

This is the only type of failure for the occurrence of which we have no evidence at all. It is quite characteristic that at the stage when e.p.p.'s are no longer evoked (see below), the spontaneous discharge of m.e.p.p.'s is still very evident.

Presynaptic failure of propagation

When one records the electrical activity of single end-plates during indirect tetanic stimulation *in vitro* or *in situ*, spikes or e.p.p.'s are seen, depending upon whether there is further propagation along the muscle fibre. If the stimulation is at a sufficiently high frequency and of sufficient duration, a new type of block is observed. The e.p.p.'s suddenly begin to fail intermittently in an all-or-nothing manner (Figs. 5, 11). This behaviour (which can be observed with intra- or extracellular electrodes) has only two possible explanations: either nerve impulses fail to reach the presynaptic terminals, or, when they do reach the terminals, they are unable to initiate the release of ACh. The presence of m.e.p.p.'s excludes the possibility that the post-synaptic membrane is only intermittently sensitive to the transmitter.

When intermittent activation was observed, it was first essential to exclude the possibility that the stimulus was close to the nerve threshold by raising the

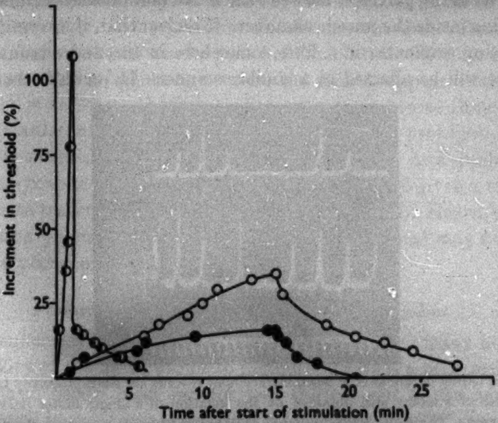

Fig. 10. Changes in the threshold of one muscle fibre caused by direct repetitive stimulation of a curarized diaphragm at three different frequencies (22° C). The electrical activity was recorded with a bipolar electrode close to the surface of the muscle. Stimulation began at 0 time, and ceased at the maximum of each curve: ●, 10/sec; ○, 13/sec; ◐, 20/sec. During the period of recovery the threshold was found with testing pulses (1/sec) applied at suitable times.

Fig. 11. Presynaptic failure of impulses *in vitro* and *in vivo*. The intracellular potentials were recorded at end-plate regions. *A*: diaphragm at 36° C; resting potential 63 mV; 10 min after beginning nerve stimulation at 44/sec. *B*: diaphragm at 36° C; resting potential 41 mV; 15 min after start of stimulation at 44/sec. *C*: gracilis anticus *in situ*; 3 min after start of stimulation at 50/sec. *D*: gracilis anticus *in situ*; resting potential 57 mV; 2 min after start of stimulation at 250/sec.

intensity of the stimulus. By monitoring the nerve action potential it was shown that there was no appreciable defect in conduction in the main nerve trunk at a time when many end-plates suffered intermittent or even complete block. However, this did not exclude failure of conduction in two portions of the nerve trunk which might be especially vulnerably placed, the portion which remains within the partition between the nerve and muscle chambers, and the portion lying inside the muscle chamber. It is clear that, if intermittent failure of conduction originates in a fibre somewhere in the nerve trunk, the entire motor unit will be affected in a similar manner. If, on the other hand, the failure occurs at a point *distal* to the most central site of branching of that fibre, the individual members of the motor unit will not behave in the same way. This argument was put to the test in experiments in which single motor nerve fibres were isolated in the phrenic nerve (for the method see Krnjević & Miledi, 1958). Simultaneous recording from two separate end-plates during a tetanus showed conclusively that members of the same motor unit do not fail in unison (Fig. 12). Evidently, the failure of propagation takes place peripherally, probably either at the first node of Ranvier beyond a division of the nerve fibre, or in the very narrow region just before the terminal expansion at the end plate.

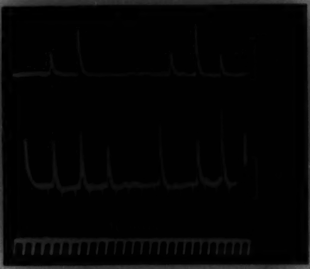

Fig. 12. Presynaptic failure as seen when intracellular records are simultaneously taken from two end-plates of a motor unit obtained by the isolation of a single motor nerve fibre in the phrenic nerve. The nerve was stimulated at 64/sec. The small potentials that can be seen in both traces were clearly distinguished as spontaneous miniature potentials when higher amplification was used.

The possibility that the nerve impulse reaches the presynaptic membrane, but fails to release ACh, was considered unlikely for two reasons. Although e.p.p.'s which are recorded from two fibres belonging to the same motor unit do not fail synchronously, we have found in several cases a definite interrelation between them (unpublished observations) which suggests that block cannot occur far from a site of branching. Further, this explanation is inconsistent with the hypothesis of a simple relationship between the release of ACh and the degree of depolarization of the nerve endings (Liley, 1956*b*).

There are very wide differences in the frequencies of stimulation at which presynaptic failure may occur in various fibres. *In vitro* and *in situ* the range may extend from over 5/sec to 100/sec, or sometimes even more. The lower figures are less common *in situ* and the higher figures less common *in vitro*. But apart from that no generalization can be made. The failure may appear quite early and then progress slowly, or it may appear late and go on to complete failure quite suddenly. Every possible variation is seen. Quite often, a steady state may be reached at a certain frequency of stimulation with a corresponding rate of failure. If the frequency is raised the failures appear more often; if the frequency is decreased the failures become rarer, or cease altogether.

One important consequence of presynaptic failures of propagation is that they relieve the constant bombardment on the end-plate. As a result, the pre- and the post-synaptic membranes and the muscle fibre are given a rest, which enables them to transmit impulses more effectively (Fig. 6). Hence, somewhat irregular action potentials (and accompanying contractions) may reappear in fibres after a period of complete inactivity.

Failure of initiation of conducted nerve impulses

At low frequencies of stimulation, this is only seen after many hours when the excitability of the nerve begins to diminish. There is a progressive rise in threshold which occurs most rapidly near the cut end of the nerve. It is especially quick in onset in preparations *in vitro* kept at 37° C.

Changes in muscle electrolytes produced by prolonged soaking and repetitive stimulation

We have studied the electrolyte composition of a small number of muscles after soaking and stimulating at 22 and 37° C (Table 1). The diaphragms which were merely soaked were left in the oxygenated Ringer–Locke solution at 22° C for 1–7 hr. There was a substantial increase in the Na content, but only a little decrease in K content. The duration of the soaking bore no relation to the final concentrations in this small sample. Intracellular concentrations were calculated on the assumption that our control and soaked muscles had the same inulin spaces respectively as the control and soaked rat diaphragms studied by Creese (1954). The intracellular concentrations of Na and K are somewhat higher than those given by Creese because of a slightly (but consistently) greater Na and K content in our muscles, but the relative changes produced by soaking are very similar. The intracellular concentrations of Cl were calculated as above. For the control muscles the plasma Cl was assumed to be 110 m-moles/kg (Conway & Hingerty, 1946).

The rapid decline in the activity of muscles kept *in vitro* at 37° C has already been mentioned. It is evidently associated with a profound change in the composition of the muscle fibres. After a 5–6 hr experiment, consisting approximately of alternating periods (10–15 min) of stimulation (at 5–40/sec) and of rest, the intracellular concentration of Na is practically that of the Ringer–Locke solution, while the intracellular concentration of K is less than one third of the control value. Muscles kept *in vitro* at about 22° C under the same conditions of stimulation remain active for a much longer time; correspondingly, it can be seen that they are able to maintain appreciable gradients of concentration across the cell membrane, even after 12 hr. There is, in fact, little difference between the Na contents of soaked muscles which have and have not been stimulated.

TABLE 1. The effects of soaking in Ringer-Locke solution, and of prolonged stimulation at two different temperatures, on the concentrations of Na, K and Cl in rat muscle

	No. of muscles	Water (%)	Concentrations in whole muscle (m-mole/kg wet weight)			Mean intracellular concentrations (m-mole/kg water)		
			Na	K	Cl	Na	K	Cl
Diaphragm								
In situ controls (means +S.D.)	7	75·9±1·5	47·7± 9·5	94·3±12·5	25·2±4·2	30	177	0·5
Soaked at 22° C	3	79·0–79·2	82·1–106	77·5– 96·6	—	98	158	—
Soaked and stimulated at 22° C	2	81·5–82·1	89·3–100	50·9– 53·7	80·2–85·2	100	92	50
Soaked and stimulated at 37° C	2	83·2–85·3	117–132	19·3– 38·2	100–119	146	50	75
Gracilis								
In situ control	1	78·1	36·5	100	25·7	—	—	—
Stimulated *in situ*	1	83·9	52·0	85·4	43·2	—	—	—

For comparison, Table 1 also gives the electrolyte composition of a gracilis muscle after an experiment *in situ* lasting about 7 hr, and also that of the symmetrical gracilis from the other leg (unstimulated control). The greater deterioration of muscles kept *in vitro* must be ascribed at least partly to impaired aerobic metabolism. Zierler (1956) found that the O_2 consumption of the isolated rat diaphragm is much depressed by handling and washing. This may result from the loss of enzyme systems and of oxidizable endogenous substrates. The effect of a lower temperature should be related to the observation that the rat diaphragm can maintain adequate function under anaerobic conditions for several hours at room temperature but not at 37° C (Ellis & Beckett, 1954).

DISCUSSION

Two features were mainly responsible for failure of neuromuscular propagation. One was block of propagation between the end-plate and the rest of the fibre and the other presynaptic failure, probably in the final ramifications of the motor nerve.

Post-synaptic failure

The first of these seems to be caused by a combination of two changes, a rise in muscle fibre threshold, and a diminution of the e.p.p. The change in threshold with fatigue has also been observed in cat muscle by Gruber (1914), Rosenblueth (1940) and Brown & Burns (1949), but not in frog muscle (Rushton, 1933). It would seem to be especially important because of the high safety factor found normally for propagation away from the end-plate. It is generally agreed that the threshold for a conducted spike at the end-plate in rats is less than 10 mV (Lundberg & Quilisch, 1953; Bryant, 1957). With such a low threshold a large decrease of the e.p.p. would be necessary for propagation block. According to Boyd & Martin (1956), the normal amplitude of the e.p.p. in cat muscle is about 40 mV: hence one would expect conduction to be maintained as long as the e.p.p. was not much less than about a quarter of its normal amplitude. However, if the threshold itself changes there could be block even with little diminution in e.p.p. We have shown that the threshold may increase several times in only a few minutes.

There is little evidence with regard to the cause of the change in threshold. We do not even know if it requires activation of the whole muscle fibre or not. It might conceivably be caused by the accumulation of K or certain chemical by-products of the contraction. However, one would not expect the removal of such by-products by diffusion from the surface of the muscle to require nearly 10 min, unless the sarcolemma behaves as a remarkably impermeable barrier. Another possibility is that so much Na enters the muscle fibre that the normal concentration gradient is considerably diminished. This may be expected to lead ultimately to inexcitability by gradually reducing the inward Na current.

The amount of Na which is required to change the membrane potential by about 120 mV can be easily calculated, taking the membrane capacity as $3\mu F/cm^2$ (Boyd & Martin, 1956); the least increment of internal Na per impulse is then 3·6 pmoles/cm^2 of surface membrane. Our observations suggest that most fibres will begin to fail to respond within 3 min after the onset of stimulation at 50/sec. We may therefore take 10,000 as the critical number of impulses. The corresponding change in concentration of Na inside a muscle fibre with a radius of 20μ would be 36 m-moles/l. of water. This calculation does not take into account a likely increase in the activity of the Na pump. It is possible that the actual gain in Na is appreciably greater than this theoretical minimum (cf. the Na gain of invertebrate nerves *in vitro*, Hodgkin, 1951). If the real increment per impulse were twice the minimum value, the change in the internal concentration would be some 70 mM. This may seem a great deal, but it should be remembered that merely soaking a diaphragm in Ringer–Locke solution causes an average increase in internal Na of the same magnitude, *without producing inexcitability*. It is, of course, possible that the accumulation of Na in the soaked muscles is mainly extracellular, but it is not clear how this can come about without a corresponding increase in the inulin space. The evidence, on the whole, does not seem to us to favour the suggestion that the inexcitability is entirely caused by an excess of Na inside the muscle fibres.

The long delay in recovery of excitability, which clearly does not depend upon the arrival of subnormal impulses (cf. Lucas, 1911), may be partly responsible for the phenomena of Wedensky inhibition. The cumulative nature of this depression is shown by the fact that the refractory period of the unfatigued muscle fibre stimulated directly (with the second stimulus about four times threshold) is only between 2·5 and 4 msec (gracilis *in situ*), which is consistent with activation at a rate of over 250/sec. Actually we have shown that few fibres will respond to stimulation at even one-fifth of this frequency for more than 1 min (see also Peruzzi & Zanghi (1952) for responses of rat diaphragm stimulated at 100/sec). The second factor, which tends to cause post-synaptic failure of propagation, is a progressive diminution of the e.p.p. during prolonged repetitive activity. Several changes may be involved, but perhaps the most important (Fig. 8) is a decrease in the sensitivity of the end-plate to ACh (cf. Katz & Thesleff, 1957). It is also possible that less ACh is released for each impulse (Rosenblueth, 1950).

Presynaptic failure

There are very few references in the literature to the kind of presynaptic failure which has been described in this paper. Dun (1955*a*) seems to be the only author to have suggested that there may be failure of conduction in the fine motor nerve terminals in normal muscles, albeit on the basis of rather unsubstantial evidence. Brooks (1954) thought that botulinum toxin probably blocks conduction in the fine branches close to the nerve endings, but more recently (Brooks, 1956) he has rejected this suggestion.

It is evident that the failure of propagation might occur at either the point of branching of the fibre, or in the region where the fibre, now very thin and no longer myelinated (Cajal, 1909), expands to form the nerve ending. On theoretical grounds one would expect the safety factor for propagation to be appreciably lower at these points, and so block would appear there when the threshold was raised. Dun (1955*b*) claims that certain discontinuities can be detected during propagation past the point of division of the sensory nerve fibres near the spinal ganglion. It is possible that the phenomenon of intermittent conduction in the dorsal columns of the spinal cord (Barron & Matthews, 1935) is at least partly caused by block in the region where collaterals are given off.

Many factors seem to be involved in the presynaptic failure; it will be shown in a later publication that the rate of intermittent failure is very sensitive to the temperature and the O_2 supply, and that failure is seen in preparations in which neuromuscular transmission has been blocked to eliminate any detrimental factors associated with contraction.

It may be noted that in a recent paper dealing with the monosynaptic reflex discharge of single motoneurones, Lloyd (1957) described failure of transmission with repetitive stimulation which resembles rather strikingly the presynaptic type of failure described here. The possibility clearly arises that an analogous presynaptic block may also restrict the transmission of repetitive activity at synapses in the C.N.S.

Contractile failure versus propagation failure

Overriding importance has been attached by Merton (1954) to failure of the contractile mechanism in fatigue. He believes that this failure occurs before any other and that it is therefore the limiting factor. His conclusion was based on the observation that even in complete voluntary human fatigue, there was no change in the electrical activity of the muscle as recorded through the skin by overlying electrodes. It is difficult to reconcile this evidence with Naess & Storm-Mathisen's (1955) assertion that electrical activity of the human muscle does diminish with fatigue, and the demonstration by Seyffarth (1940) that as the tension falls in fatigue the frequency of discharge of individual units recorded by electromyography also diminishes, and the total number of active units is reduced. One can only suppose that the method of recording did not give a picture representative of the activity of the whole muscle.

The most direct method of demonstrating failure of the contractile mechanism is to record simultaneously the electrical signals and the tension produced by isolated muscle fibres. This has been done in at least two different laboratories, but unfortunately with conflicting reports. Ramsey & Street (1941, 1942) concluded that contractile failure may never occur and that graded failure in the whole muscle is caused by a successive decrease in the number of responses and of active fibres. Steiman (1943) also found evidence of a similar failure at a relatively high frequency of stimulation; however, when the frequency of stimulation was only 5/sec, there was obvious evidence of a progressive diminution in contractions without failure of propagation. He concluded that the type of fatigue actually observed in any one case would depend upon the rate of excitation, as suggested by Rosenblueth (1950).

There are at least two possible explanations why different conclusions were reached. One is that Ramsey & Street did not investigate the effects of a sufficiently slow tetanus. The other is based on an important difference in the respective experimental techniques. The isolated fibres were kept in Ringer's solution by Ramsey & Street and in paraffin by Steiman. It is conceivable that the contractile fatigue observed by the latter was caused by anoxia or the accumulation of K (cf. the demonstration by Heron, Hales & Ingle (1934) that rat muscle *in situ* shows little loss of contractile power when stimulated directly at 3/sec for 10–17 *days*). The issue cannot be decided conclusively until these experiments are repeated.

Our observations showed clearly that the failure of propagation seen in isolated muscles occurs readily in whole muscles, even *in situ*. It is possible that some degree of contractile fatigue was also present in our experiments; however, we did not find action potentials in muscles which were not contracting at all. There was no evidence of complete 'uncoupling' between the action potential and the contractile mechanism (cf. Merton, 1956).

Highest frequencies of activity observed in mammalian muscle

It has been shown by several authors that in voluntary contraction there is a progressive increase in the number of active motor units, and, over a certain range of tension, also an increase in the frequency of discharge of each unit (Adrian & Bronk, 1929; Smith, 1934; Seyffarth, 1940; Denny-Brown, 1949; Bigland & Lippold, 1954). These authors also agree in stating that units in normal muscle do not fire at frequencies much above 20–50/sec, even for comparatively short periods. In paretic human muscle a wider range of frequencies is found, and the maximum may extend up to 70–80/sec (Seyffarth, 1940). This may indicate a functional adaptation to the greater load on the surviving motor units. When fibres give a maintained tetanic discharge, the frequency is quite low. The longest steady sequence seen by Smith (1934) in voluntary contractions of human muscle lasted 13 min: its frequency was only 9/sec.

It is clear that muscle fibres usually function at frequencies which, in the light of our experiments, are consistent with sustained activity. Can this be because the two types of peripheral failure described here also occur in prolonged voluntary effort, and thus set a ceiling to the frequency of discharge of the muscle fibres in the region of 50/sec?

It is common knowledge that the movements of tired muscle are rather coarse and accompanied by tremor. Seyffarth (1940) and Bigland & Lippold (1954) have shown that the electrical units under these conditions tend to fire much more irregularly than normal. This could be explained by the occurrence of an intermittent, irregular type of failure of propagation as well as by a reduction in the total number of active units. However, we must remember that peripheral failure is only likely to occur during a sustained tetanic activity of the motoneurones; if the C.N.S. is capable of maintaining a discharge at a frequency above 50/sec, then peripheral failure must be expected. On the other hand, if an equally weak link exists in the spinal cord, this may cause a central failure which will protect the periphery (Waller, 1891; Reid, 1929). As far as the periphery is concerned, tremor and intermittent activity might also be evident, but these should be much coarser since entire motor units would be affected.

We have not much evidence about the maximum frequency of discharge of motoneurones under reasonably normal conditions. According to Adrian & Bronk (1928, 1929) the rate of firing of motoneurones in rabbits and cats may be as high as 90/sec, but such high frequencies only seem to have been observed for a short time. Lloyd (1957), on the other hand, observed depression of the monosynaptic reflex in cats at frequencies greater than 3/min. Eccles, Eccles & Lundberg (1957) believe that the duration of the phase of after-hyperpolarization determines the frequency of firing of motoneurones subjected to continuous synaptic bombardment. It is well known that the strongest voluntary maintained contraction declines as soon as the peak tension is reached (Reid, 1929; Seyffarth, 1940; Merton, 1954) and that there is little relation between the maximal pull exerted and the subjective feeling of maximal effort (Dessy & Grandis, 1904; Merton, 1954). These observations seem consistent with the possibility that the effectiveness of the subjective effort is limited by either central or peripheral failure of propagation.

SUMMARY

1. A study has been made of the mechanism of neuromuscular failure during tetanic stimulation in rats, *in vitro* and *in situ*.

2. An intracellular electrode may cause failure of propagation along a contracting muscle fibre because of local damage.

3. Repetitive activity is limited by failure of e.p.p.'s to initiate propagated impulses in muscle fibres because the e.p.p. diminishes and the threshold of the muscle fibre increases. The post-synaptic membrane apparently becomes less sensitive to ACh.

4. As a result of (3), muscle fibres cannot respond for more than a few minutes at frequencies greater than about 50/sec. If excitation is maintained at a frequency not much greater than 10/sec the likelihood that propagation will fail is small.

5. Another limit is set upon tetanic activity by the occurrence of presynaptic failure of propagation, probably in the fine terminal filaments of the motor nerve. There is a wide difference between fibres with regard to the duration and frequency of stimulation needed to cause this type of failure.

6. Intermittent presynaptic failure of propagation tends to protect the muscle fibre from excessive stimulation and to permit a fatigued muscle fibre to resume activity at a lower frequency.

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THE REPRESENTATION OF THE SPLANCHNIC AFFERENT PATHWAYS IN THE THALAMUS OF THE CAT

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As a result of the work of Tower (1933) and of Gernandt & Zotterman (1946) it is now well known that the gut is supplied with its own sensory system, in many respects similar to that of the skin, except that its distribution is sparse and it contains a smaller proportion of large fibres. In the cat, the large fast conducting afferent fibres of the splanchnic nerve are situated mainly in the dorsal columns and the small slowly conducting fibres mainly in the ventrolateral columns of the spinal cord (Amassian, 1951*b*; Downman & Evans, 1957). Downman (1951), Amassian (1951*a*) and Gardner, Thomas & Morin (1955) have demonstrated that the fast conducting fibres project to the trunk region in the somatic sensory cortex, although the evidence for the projection of the slowly conducting fibres is conflicting. In regard to the representation of the splanchnic afferents in the thalamus little work has been done. Aidar, Geohegan & Ungewitter (1952) have recorded responses to stimulation of the splanchnic nerve in various parts of the brain stem and thalamus and Patton & Amassian (1951) found evidence that the fast conducting fibres relayed in the posterior ventral nucleus. These earlier investigations had been conducted using large steel recording electrodes, and in this present work smaller electrodes have been used in order to record from single neural units following electrical stimulation of the splanchnic nerve. The object has been to determine the positions from which responses can be obtained in the thalamus and to relate each unit to the type of nerve fibre which is required to activate it, in the hope of deriving information about the relay of both fast and slowly conducting fibres. In addition, an attempt has been made to activate each of these units by stimulating an appropriate area on the body surface, for it has for long been supposed that the phenomenon of referred pain is due to a convergence of somatic and visceral pathways at some level in the nervous system and it was thought that this approach might shed some light on this old problem.

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A preliminary account of some of this work has already been given (McLeod, 1956).

METHODS

Animals. All but one of the experiments were conducted on cats weighing between 2 and 3 kg. In most cases they were anaesthetized with intraperitoneal pentobarbitone (Nembutal, Abbott Laboratories, 35 mg/kg), but intraperitoneal allobarbitone (Dial, Ciba, 0·5 ml./kg), chloralose (80 mg/kg) and ether were also employed as anaesthetics in some experiments. A baboon (11 kg) was used in one experiment and was anaesthetized with intraperitoneal pentobarbitone (35 mg/kg).

Preparation of the animal. The left splanchnic nerve was exposed retroperitoneally through an incision in the flank. In order to record the action potential of the nerve after conduction over a distance of suitable length, the seventh, eighth and ninth ribs were resected and the sympathetic trunk freed from the overlying pleura and surrounding connective tissue. The rami communicantes were not cut, and the part of the sympathetic chain between the stimulating and recording electrodes was left undisturbed beneath the pleura. Once the thorax had been opened, the cat was kept alive by artificial respiration with a respiratory pump. A portion of the calvarium was removed, after which the dura mater was reflected to allow for insertion of the recording micro-electrode into the thalamus, and the cortex was then covered with paraffin to prevent drying.

The cat was then mounted in an animal holder of the design described by Gaze & Gordon (1954) with its left side uppermost and its head aligned in Horsley–Clarke planes. The body cavity was filled with warm paraffin, the temperature of which was kept constant at 38–40° C by means of electric heaters. A pair of stimulating electrodes made of silver wire were placed beneath the distal part of the splanchnic nerve and a pair of similar recording electrodes beneath the thoracic sympathetic trunk at a distance of about 4 cm from the stimulating electrodes. In order to assist in reducing the size of the shock-escape, the nerve was earthed between these two pairs of electrodes. The deep body temperature of the cat was maintained at 38–40° C throughout the experiments by means of direct radiation from a shielded electric heater. The technique was essentially similar throughout in the experiment on the baboon.

Micro-electrodes. Each consisted of a Pyrex glass micropipette drawn out to a fine tip of external diameter about 10μ, through which fine Wollaston or silver wire of tip diameter $2–5\mu$ was inserted until it just projected beyond the glass tip. The length of the micro-electrode shaft was about 25–35 mm and the resistance of the micro-electrode was 1 MΩ or less. The micro-electrode was used as a unipolar electrode and the indifferent electrode consisted of an earthed silver plate inserted into the temporal muscle.

Electronic apparatus. The micro-electrodes led through a cathode follower (Bishop, 1949) into a four-stage push-pull R–C-coupled amplifier of over-all time constant 3 msec. The action potential of the nerve was similarly amplified, but in this case a cathode follower was not employed. This display unit consisted of three cathode ray tubes arranged vertically one above the other. The responses from the thalamus were led to the plates of one tube, the nerve action potential to those of another, and a time marker to those of the third. Being driven from the same time base unit, the sweeps of all three tubes were synchronized and the camera was arranged so that they could all be photographed together. It was thus possible to compare the action potential of the nerve with the thalamic response to the same stimulus. The output of the amplifiers was monitored on a loudspeaker.

The nerve was stimulated from a battery-operated stimulator giving a condenser-like discharge of half-time about 0·5 msec, and this was led through a radio-frequency-coupled unit, isolated from earth (Schmitt & Dubbert, 1949). In many cases the skin was stimulated through two closely juxtaposed needles to which the output of a second stimulator was coupled by a 1:1 transformer.

Histological procedures. The micro-electrodes were always inserted in Horsley–Clarke coordinates, and tracks were never placed closer together than at intervals of 0·5 mm. After each experiment the positions from which the responses had been recorded were determined histologically, and to facilitate the sectioning of the block in Horsley–Clarke planes indian ink guide marks were made in the brain at the end of each experiment (Gaze & Gordon, 1954). The brain was perfused and hardened in 10% formaldehyde, a block of the relevant part cut, washed, dehydrated and embedded in low-viscosity nitrocellulose (Chesterman & Leach, 1949). When embedding was completed, 50μ serial sections were cut from the block and these were stained with Heidenhain's haematoxylin, except for every tenth section which was stained with cresyl violet so that nuclear structures would be visible.

The relevant sections were enlarged and printed directly on photographic paper. The depths of the needle tracks were measured on the prints, the shrinkage factors determined and the positions from which the responses had been obtained calculated, marked on the photographs and related to surrounding nuclear structures. Composite maps were prepared, incorporating the results of a number of experiments on different cats. These were traced from sections taken at 0·5 mm intervals through the appropriate region of a well stained brain of average size, and the positions of the responses were marked on these maps. It has been estimated that the error in localizing the position of the recorded response is less than 0·5 mm with this technique (Gaze & Gordon, 1954), but there is a further loss of accuracy in transferring these positions to the schematic maps.

Fibre counts of splanchnic nerves. About 2–3 cm of the left splanchnic nerve was removed from the animal, cleaned of excess fatty tissue and tied to a glass rod. The nerve was stained in 0·5% osmic acid for 36 hr, after which it was washed, dehydrated and embedded in wax. Serial sections were cut to 5μ and mounted. The best section was photomicrographed, enlarged and printed. The total magnification was ×2125 and each fibre was measured on the print with a rule in its largest and smallest diameters, the mean of which was taken. Measurements were made to the nearest 0·25μ and from these the fibre spectrum of the nerve was constructed.

General experimental procedure. The splanchnic nerve was electrically stimulated at the rate of about once every two seconds at sufficient strength to activate all the myelinated fibres. The microelectrode was slowly lowered through the brain in the predetermined co-ordinates, and when a unit response was found which consistently followed each stimulus applied to the nerve its threshold was determined in terms of the fibre groups in the nerve which were required to activate it. Since strong stimulation of the nerve gives rise to reflex muscle twitching it was necessary to eliminate the possibility of the thalamic responses being due to the stimulation of cutaneous or deep somatic afferent pathways by reflex movement. For this purpose, muscular paralysis was produced by intravenous injections of either 2·5 mg of tubocurarine or 1·0 mg of decamethonium iodide when a unit response was located.

In many experiments an attempt was made to activate the splanchnic units by stimulating the body surface, e.g. by moving hairs or by pinching the skin. In order to demonstrate that it was the same unit responding in either case, the appropriate region of the skin was then stimulated electrically through one channel of the stimulator, and the splanchnic nerve through the other. The interval between the two stimuli was gradually reduced until the response to the second stimulus was blocked by that to the first. This phenomenon of blocking below a certain time interval, together with similarity in the configuration of the two unit responses, was taken as a criterion of convergence of the two central pathways.

Notation. Where relevant, mean figures are expressed with their standard errors.

RESULTS

The compound action potential of the splanchnic nerve

It was necessary to investigate the features of the compound action potential of the splanchnic nerve at the outset, because the threshold of each thalamic unit was determined by reference to it.

When the splanchnic nerve is maximally stimulated and its action potential recorded with bipolar electrodes over a conduction distance of about 4 cm it can be seen to consist of four main groups of waves which are present in all cats (Text-fig. 1):

(1) The first wave is of large amplitude and very low threshold, and it often exhibits a hump representing a second fast wave. It has a maximum conduction velocity of 70–75 m/sec, and its threshold, refractory period, and velocity of conduction are all consistent with its representing activity in A fibres.

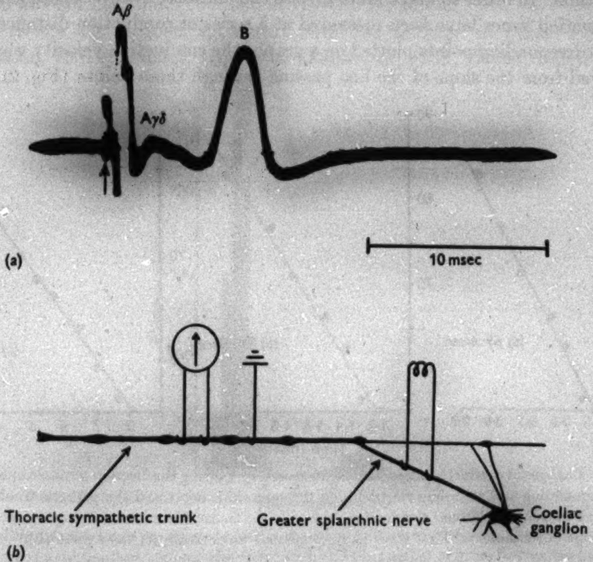

Fig. 1. (*a*) Compound action potential recorded with bipolar electrodes from the sympathetic trunk of a cat at a conduction distance of about 4 cm, following electrical stimulation of the splanchnic nerve at arrow ↑. The C wave is not visible in this record. Anaesthetic: sodium pentobarbitone. (*b*) The arrangement of the electrodes on the splanchnic nerve and sympathetic trunk used throughout these experiments.

(2) After the first main wave, and with a stronger stimulus, there appears a group of small deflexions whose threshold is above the stimulus strength required to activate the first wave maximally. It has a maximum conduction velocity of 30–35 m/sec, and, like the first wave, has properties consistent with its representing activity in A fibres.

(3) A third wave appears at about the same threshold as the second group of waves, but it is only submaximally excited at the maximum for the second group. It is of longer duration than the other waves, and its amplitude is

usually comparable to that of the first wave. It has a maximum conduction velocity of 10–12 m/sec, and its properties are consistent with those of B fibres.

(4) At much greater stimulus strengths and at a higher gain, a fourth wave of small amplitude can be seen. It has a maximum conduction velocity of 1–2 m/sec, and has properties consistent with those of C fibres.

The conduction velocities of these waves have been calculated in a large number of experiments from single values of conduction distance and conduction time. In order to make more precise calculations, in two experiments the conduction times have been measured at a series of conduction distances and the corresponding points plotted on a graph; the conduction velocity was then derived from the slope of the line passing through these points (Fig. 2).

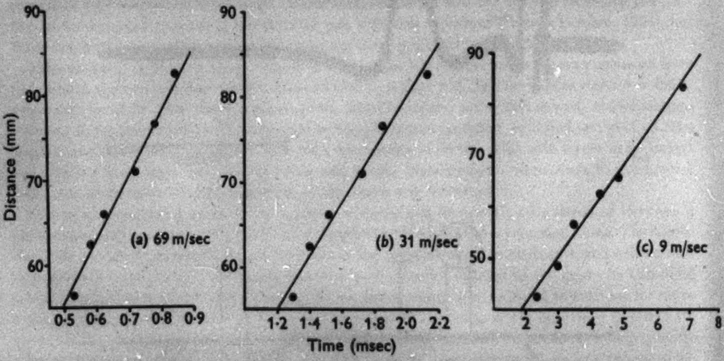

Fig. 2. Changes in conduction time (abscissae) caused by varying the distance (ordinates) between stimulating and recording electrodes on the splanchnic nerve and sympathetic trunk. Cat: sodium pentobarbitone. Conduction velocity of the fastest fibres in each group is given by the slope of the line. (*a*) First wave (Aβ); (*b*) second wave (A$\gamma\delta$); (*c*) third wave (B).

Gernandt & Zotterman (1946) studied the responses of single nerve fibres in the mesenteric nerves of the cat when the gut and mesentery were stimulated by various means, and on the basis of the differing spike sizes of the responses they divided the sensory fibres into three main groups (Aβ, Aδ, and C), similar to the groups of cutaneous nerves, a classification which subsequent workers have followed. Support is lent to this classification by comparing the compound action potential with the fibre spectrum (Fig. 3). There is a group of fibres in the 8–13μ range which is responsible for the first wave, a small group of fibres in the intermediate range which gives rise to the second wave, and a large group of fibres in the 1–3μ range responsible for the large third wave. The fact that the electrical properties of the third wave are similar to those of fibres in the B group is in conformity with the finding of Ranson & Billingsley (1918) that nearly all the preganglionic efferent fibres in the splanchnic nerve were in the range 1–3μ. Examination of the splanchnic nerve confirms that large groups of unmyelinated fibres are present (Pl. 1).

It therefore seems reasonable to summarize the main features of the compound action potential of the splanchnic nerve as follows:

(1) An Aβ wave of maximum conduction velocity 70–75 m/sec,
(2) an A$\gamma\delta$ wave of maximum conduction velocity 30–35 m/sec,
(3) a B wave of maximum conduction velocity 10–12 m/sec, and
(4) a C wave of maximum conduction velocity 1–2 m/sec.

These results are in good agreement with the findings of other workers (Amassian, 1951*b*; Baïsset, Laporte & Montastruc, 1952; Widén, 1955).

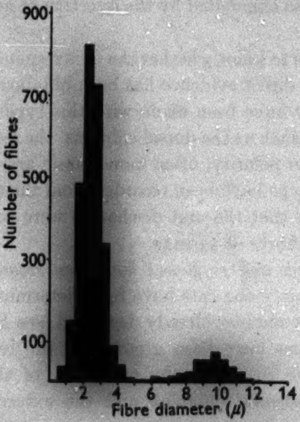

Fig. 3. Size distribution of myelinated fibres in the splanchnic nerve of the cat. The left splanchnic nerve of a cat has been stained with osmic acid, and the number of fibres (ordinates) has been plotted against the total fibre diameter (abscissae) measured to the nearest 0·25μ.

The nature of the thalamic unit response

The conventional criteria of all-or-nothing response, and uniform spike amplitude and spike configuration, have been employed for identifying single unit activity in the thalamus, and those responses recorded following splanchnic stimulation were usually of amplitude 300–500μV and could be recorded over a depth of 200–300μ. When the micro-electrode was in the optimal recording position, the great majority of the spikes were triphasic and predominantly negative in polarity with small initial and terminal positive phases, and were often superimposed on a small negative wave. However, sometimes the spikes were diphasic or monophasically negative. Very frequently the unit response consisted of only one spike, but sometimes there was a train of spikes, the maximum number to have been consistently present in a single unit discharge having been six. Adrian (1941) and Gaze & Gordon (1954) have described an intermittent thalamic after-discharge, particularly under Dial, but this has not been observed in this work.

It was important to establish that the thalamic unit response was a direct result of activity in the splanchnic afferent pathways and was not due to the spread of stimulus to surrounding structures with consequent activation of somatic afferent pathways. This was shown by crushing the nerve proximal to the stimulating electrodes, causing the thalamic response to be abolished, and then moving the electrodes proximal to the crush when the thalamic response returned. Any possibility that the thalamic responses were initiated by reflex muscle movements was eliminated by the injection of neuromuscular blocking drugs.

It would be of value to know whether the unit responses were recorded from cells or fibres, but no direct evidence has been obtained on this point. However, when recordings have been made with this type of electrode from undoubted fibre regions such as the dorsal columns, the responses have been predominantly positive in polarity, often monophasic and smaller in amplitude. No responses of this type have been recorded from the thalamic nuclei, and it therefore seems likely that the unit discharges were recorded from synaptic regions and not from fibres of passage.

Positions from which unit responses have been recorded. The positions of ninety-five units in thirty-one cats have been determined and plotted in the composite maps in the manner already described (see Methods). In all but a few cases the responses have been grouped in the region of the posterior ventral nucleus of the thalamus, the remainder of the units having been recorded from the reticular complex and the external medullary lamina (Fig. 4*a*–*d*).

In each experiment a systematic search has been made over the ventrolateral region of the thalamus from which tactile responses can be recorded, and therefore the maps give some indication of the density of the representation of the splanchnic afferents in this part of the thalamus. Responses have been recorded more frequently in the lateral parts of the posterior ventral nucleus than in the medial, and although they are scattered throughout this nucleus they tend to be concentrated more in the central regions than at the rostral or caudal ends. Although the micro-electrode has passed consistently through the lateral nucleus, no responses were recorded from this nuclear mass. Other parts of the thalamus have not been investigated systematically, but the micro-electrode has frequently passed in planes anterior and posterior to those shown in the maps without ever recording responses from the groups of thalamic nuclei in those regions. No search was made in the mid-line region.

Every time that a unit which responded to stimulation of the splanchnic nerve was isolated, the skin region from which tactile responses could be elicited at this recording side was investigated. Only two units were found in places which did not also respond to tactile stimulation, and these both fell in the reticular complex. The majority of responses were in the area of representation of the abdomen and trunk, but they were also scattered in the forelimb and hind limb regions. No responses were recorded from areas responding to stimulation of the face and neck, but a few units were recorded in the ta[illegible]. Thus, although most of the splanchnic afferent responses have been obtained from the regions of representation of the trunk, there is considerable overlap into adjacent somatic areas.

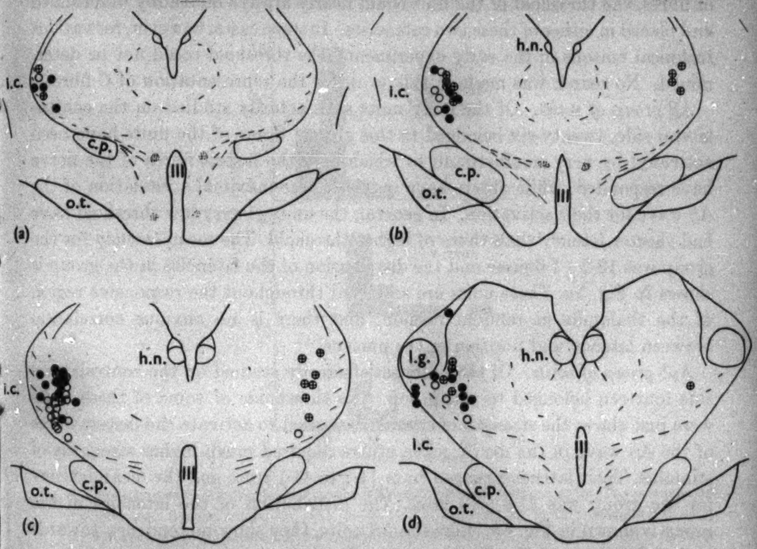

Fig. 4. Four consecutive composite maps from the cat's brain, showing the positions from which single unit responses were recorded. The left-hand side in each map is contralateral to the stimulated nerve. The maps are traced from coronal sections cut in Horsley–Clarke frontal planes at 0·5 mm intervals in the anteroposterior plane. (*a*) is the anterior map of the series. The main features on the maps are indicated as follows: c.p., cerebral peduncle; i.c., internal capsule; h.n., habenular nucleus; l.g., lateral geniculate body; o.t., optic tract; III., third ventricle. The types of single unit responses, classified in terms of the fibre groups in the splanchnic nerve required to activate them, are indicated as follows; ●, Aβ unit; ⊕, Aγδ unit; ○, threshold undetermined.

Types of splanchnic unit found in the thalamus

Whenever a unit was located in the thalamus, the strength of stimulus being employed to activate it was reduced until its threshold had been reached. Since the stimulus required to activate the A$\gamma\delta$ group in the action potential was greater than that required to produce a maximal response from the Aβ group of fibres, the threshold of the unit could nearly always be readily determined and placed in either of these two categories. In some cases, however, for various technical reasons in the early experiments, the threshold could not be determined. No search was made in this work for the representation of C fibres.

Aβ *group of units*. Of the forty units satisfactorily studied on the contralateral side, twenty-six belonged to this group. Some of the units have been activated by very small stimuli to which only the fastest fibres of the nerve have responded, while others have required near-maximal stimulation of the Aβ wave for their activation. In general, the units of very low threshold have had shorter latency than those of higher threshold. The mean latency for the group was 12·2 ± 1·0 msec and the distribution of the latencies in the group is shown in Fig. 5*a*. These units are scattered throughout the responsive region of the thalamus in random fashion, and there is no obvious correlation between latency and position in the nucleus.

A$\gamma\delta$ *group of units*. Of the units satisfactorily studied on the contralateral side fourteen belonged to this group. The thresholds of some of these units were just above the strength of stimulus required to activate the fastest fibres of the Aβ wave in the nerve, while others required much higher strengths of stimulus. Their latencies ranged from 11·7 to 44·1 msec and the mean latency for the group was 21·4 ± 2·1 msec. The distribution of the latencies in the group is shown in Fig. 5*b*. Like the Aβ units, they show no tendency towards a spatial pattern of distribution within the thalamus.

Ipsilateral representation. In a series of seven cats ten units have been isolated on the ipsilateral side. All these units fell in the region of the posterior ventral nucleus, and their positions are indicated on the maps. One unit was in the forefoot region, one in that of the hind leg, and the others in that of the trunk. In all cases the tactile responses were elicited from the contralateral body surface. All the units recorded have been activated by the A$\gamma\delta$ group of afferent fibres only and their latencies have ranged from 9·0 to 27·8 msec with a mean latency of 18·5 ± 1·7 msec. The distribution of the latencies of these units is shown in Fig. 5*c*.

Convergence of splanchnic and cutaneous pathways

When a unit was located which responded to splanchnic stimulation, an attempt was made to activate the same unit by tactile stimulation of the body surface. If the unit response to stimulation by either means had the same spike configuration, and if interaction between the two pathways could be demonstrated by blocking the response to one by previously stimulating the other at a suitable time interval, the criteria for convergence of the two pathways on the same thalamic unit were taken to be fulfilled (Fig. 6). This type of interaction has been described by Amassian (1952) as occlusion.

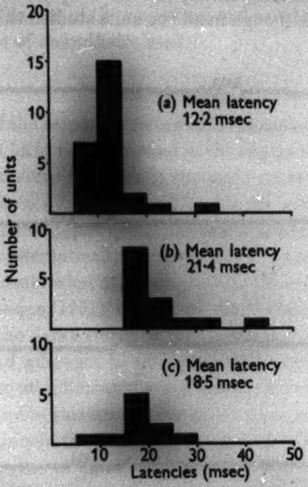

Fig. 5. The distribution of latencies among the different classes of thalamic units. The latencies are grouped into 5 msec limits, i.e. 0–5, 5–10, 10–15, etc. (*a*) Aβ units (contralateral side); (*b*) A$\gamma\delta$ units (contralateral side); (*c*) A$\gamma\delta$ units (ipsilateral side). It was not possible to determine the latencies of all the units whose positions are plotted on the maps in Fig. 4; and therefore the numbers of units referred to in Figs. 4 and 5 do not correspond in all cases.

Convergence of the splanchnic and cutaneous afferent pathways has been observed in the thalamus on the following classes of units:

(*a*) Ten out of fifteen Aβ units studied on the contralateral side. Of these, the relevant skin area has been the trunk region in four cases, forelimb in three, hind limb in two and tail in one.

(*b*) Seven out of nine A$\gamma\delta$ units studied on the contralateral side. In all cases the relevant skin area has been in the trunk region.

(*c*) Two out of six A$\gamma\delta$ units studied on the ipsilateral side. In both cases, the relevant skin area was in the contralateral trunk region.

(*d*) Two out of three A$\gamma\delta$ units studied on the contralateral side in a baboon. For one the relevant skin area was the forelimb and for the other the trunk.

In studying interaction it was found that as the interval between the conditioning and the test stimulus was gradually reduced the frequency with which the unit responded to the test stimulus diminished progressively until finally the unit ceased to fire at all. At the same time the latency of the test response increased until complete occlusion occurred. The interval at which complete occlusion occurred in any one unit depended upon which pathway was stimulated first, although in all the units studied this value fell within the range of 30–175 msec. Some units were found which responded regularly to stimulation of one pathway, but only intermittently to stimulation of the other. The type of skin stimulus required to activate the thalamic units has always been light touch or brushing of hairs, and no units were found which were activated by only pinching or pressure. The latencies of the responses to skin stimulation have varied between 8 and 30 msec.

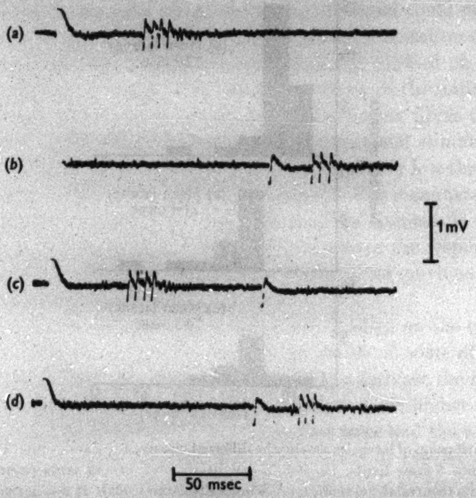

Fig. 6. Activation and occlusion of a thalamic unit by stimulating splanchnic and cutaneous afferent pathways. Cat: sodium pentobarbitone. (*a*) Response of thalamic unit to single-shock stimulation of the Aβ fibres in the splanchnic nerve. (*b*) Response of the same unit to electrical stimulation of the skin of the forefoot. (*c*) The response to stimulation of the skin is blocked by a previous response to stimulation of the splanchnic nerve. (*d*) Activation of the thalamic unit by the skin stimulus occurs when the response to previous stimulation of the splanchnic nerve has failed.

Effects of anaesthetics

In order to avoid generalizing from results which may be peculiar to one type of anaesthetic a variety of anaesthetics has been used in this investigation. Most of the experiments have been performed under pentobarbitone, but Dial, chloralose and ether have also been employed. There appeared to be no significant difference in the nature of the responses, and occlusion was observed with all the different types of anaesthetic used.

DISCUSSION

The great majority of the responses recorded in the thalamus following splanchnic nerve stimulation have been recorded in the region of the posterior ventral nucleus, and it would seem that this is the main nucleus for the relay to the cortex of the splanchnic afferent as well as for the cutaneous afferent pathways. It should be stressed, however, that this is the only region of the thalamus which has been systematically explored and it is not possible to say on the basis of these experiments what other projections the splanchnic afferents may have. Patton & Amassian (1951), using gross electrodes, recorded fast waves of latency 5–6 msec in the pars externa of the posterior ventral nucleus following stimulation of the Aβ afferents in the splanchnic nerve, but did not record activity in response to stimulating the A$\gamma\delta$ fibres. A similar representation of the Aβ fibres was found in this nucleus by Aidar *et al.* (1952) who also recorded a few slow waves, mainly in the hypothalamus and caudal part of the thalamus, which were probably due to activity in fibres of passage. By employing the technique of recording from single neural units it has been possible to show in this work that both the Aβ and A$\gamma\delta$ fibres are represented in the contralateral posterior ventral nucleus, and some A$\gamma\delta$ units have been recorded also in a similar position on the ipsilateral side. The two classes of unit were distributed in random fashion in the thalamus, although the pathways of the Aβ and the A$\gamma\delta$ fibres are well separated in the cord (Downman & Evans, 1957). A similar random distribution was found in the case of units responding to cutaneous stimulation (Gaze & Gordon, 1954) and is to be expected on anatomical grounds, for the medial lemniscus and spinothalamic tracts interdigitate in the posterior ventral nucleus (Chang & Ruch, 1947; Getz, 1952). The impression was gained during these experiments that the splanchnic units of both classes were very sparsely distributed throughout the thalamus by comparison with the dense representation of the units which responded to cutaneous stimulation alone.

All the units recorded on the ipsilateral side have been in the region of the posterior ventral nucleus, and all have been activated by the A$\gamma\delta$ fibres only. The series is too small to exclude the possibility of Aβ units being present on the ipsilateral side, but it is clear that there is a greater preponderance of A$\gamma\delta$ units on this side than on the contralateral (where the Aβ units outnumber the Aγδ units in the ratio two to one) and this is similar to the case of units which respond to different types of cutaneous stimulation (Gaze & Gordon, 1954). The presence of units on the ipsilateral side responding to stimulation of the Aγδ afferents is to be expected from a knowledge of their central pathways, for Amassian (1951*b*) and Downman & Evans (1957) have recorded activity in these fibres bilaterally in the ventrolateral columns of the spinal cord, and substantial anatomical evidence has been provided by Chang & Ruch (1947) and Getz (1952) that the spinothalamic tracts from each side of the cord are bilaterally represented in the thalamus.

It has not been possible in this work to determine the natural stimulus required to activate the thalamic units, nor to locate the sensory end organs in the gut. Edgeworth (1892), Gernandt & Zotterman (1946) and Downman & Evans (1957) have all shown that at least some of the large fibres are connected to Pacinian corpuscles, and Sheehan (1932) has estimated that there are between 50 and 150 of these in the cat's mesentery. The Aβ wave is produced by the activity of fibres in the 8–13μ range and in this study 336 of these were counted in a single splanchnic nerve, so that, if the figures are accurate, only a small proportion of fibres of this group can be connected to Pacinian corpuscles. The function of the large fibres in the splanchnic nerve is unknown, and consequently the significance of the Aβ representation in the thalamus and cortex is purely speculative. It is certain that the large fibres play no part in nociceptive reflexes, and so it is unlikely that they are responsible for conducting impulses from the gut which give rise to painful sensations. The Aγδ afferents, on the other hand, are responsible for nociceptive reflexes of many kinds. Gernandt & Zotterman (1946) found that these fibres were made active by such stimuli as pinching, squeezing, pressure and contractions of the gut in the cat, and Tower (1933) reports similar findings in the frog. The finding of representation of Aγδ fibres in the thalamus suggests that they may be responsible for carrying impulses which evoke sensations of pain. It is certain that impulses giving rise to painful sensations are conducted in the splanchnic nerves, for direct stimulation in the human subject gives rise to pain (Leriche, 1937) and pain sense from the abdominal viscera is abolished by splanchnicectomy (Bentley & Smithwick, 1940; Ray & Neill, 1947). Evidence that impulses giving rise to painful sensations are conducted in the vagus is inconclusive, and both myelinated and unmyelinated vagal afferents appear to be attached to stretch receptors in the gut wall and probably play a part in mediating the sensations of hunger and satiety (Iggo, 1955, 1957).

Interaction between the somatic and splanchnic afferent pathways has been investigated at the cortical level by Amassian (1952), who found that the evoked cortical response to stimulation of the Aβ afferents was blocked by such means as previously tapping the forepaw or stimulating a peripheral nerve. In addition, interaction had been reported at the thalamic level by Patton & Amassian (1951) who recorded summed activity in the posterior ventral nucleus and found a reduction in the size of the response to stimulation of the Aβ splanchnic afferents if a somatic afferent nerve was previously stimulated. In this work it has been shown that a large proportion of both Aβ and Aγδ splanchnic units can be occluded by stimulating a cutaneous afferent pathway, and therefore sensory pathways from the skin and abdominal viscera must converge and overlap to a considerable extent at or below the level of the thalamus. The primary site of convergence has not been determined in this work, although there is some evidence which bears on this point. Responses from a number of Aβ units were recorded from the dorsal columns in the cervical region, and in no case was occlusion observed on stimulating the appropriate skin area (G. Gordon and J. G. McLeod, unpublished observations). Also, Patton & Amassian (1951) report having found only slight convergence with Aβ afferents in the region of the medial lemniscus and dorsal column nuclei so that it seems probable that in the case of the Aβ afferents, most of the convergence takes place in the thalamus. In regard to the Aγδ fibres, Widén (1955) found convincing evidence that some interaction took place with afferents from the intercostal nerves in the spinal cord. Interaction at this level seems very likely on anatomical grounds, for the Aγδ afferents are conducted in the ventrolateral columns by means of short relays and many synapses.

Sinclair, Weddell & Feindel (1948) suggested that a sensory axon may divide peripherally, supplying both somatic and visceral structures, and they offered this as an explanation of the clinical phenomenon of referred pain. The occlusion of thalamic units which occurs when cutaneous and splanchnic afferent pathways are stimulated is very unlikely to be due to this effect, for the unresponsive time of the unit depends on which pathway is stimulated first, and, in addition, the response to stimulation of one pathway may be intermittent when the response to the other is regular. If the two pathways were common to each other, these effects could not be explained without employing a complex theoretical argument which invoked the presence of inhibitory fibres; moreover, these effects suggest that convergence takes place beyond the first synapse.

Sturge (1883) first suggested some type of convergence as an explanation for referred pain, and this theory was developed in various ways by subsequent writers. Hinsey & Phillips (1940) first expressed these theories of interaction in precise neurophysiological terms, and it is now widely accepted that to explain referred pain adequately some central convergence of the cutaneous and visceral pathways must be postulated. The visceral afferent fibres most likely to carry impulses evoking painful sensations are the fibres of the Aγδ and the C groups, and the interaction observed at the thalamic level between the Aγδ splanchnic fibres and the cutaneous pathways suggests that this may form a possible neurological basis for the reference of pain, although the type of fibres involved in the skin afferents has not been determined in this work. It is to be expected that impulses travelling up the secondary pathway common to both the skin and the viscus will be interpreted in consciousness as having come from the skin, since this is their most frequent source of origin and localization is far more exact on the body surface than in the viscera. Once a sensation arising from a viscus can be projected to the body surface the presence of sensory fibres in a cutaneous region is not essential to the reference of pain to that region, although volleys from the skin which do not themselves reach the threshold of sensation can lower the threshold of the visceral sensation (Doran & Ratcliffe, 1954). That all visceral pain is not referred can be explained on the basis of the finding that the splanchnic units were not universally occluded by stimulating cutaneous pathways, and therefore separate pathways probably exist for the projection of splanchnic fibres to the thalamus.

SUMMARY

1. Single neural units responding to electrical stimulation of the greater splanchnic nerve have been recorded extracellularly in the thalamus of the cat with micro-electrodes of tip diameter 5–10μ, and their positions have been determined histologically.

2. The responses were obtained from the region of the posterior ventral nucleus on the contralateral side, and a few have also been obtained from that on the ipsilateral side. Most of the units were recorded in regions of the nucleus which responded to stimulation of the trunk, but many were also recorded in the limb and tail areas.

3. The units on the contralateral side fell into two main groups:

(i) Units which responded to stimulation of the Aβ fibres in the splanchnic nerve, and which had a mean latency of 12·2 ± 1·0 msec.

(ii) Units which responded to stimulation of the A$\gamma\delta$ fibres in the splanchnic nerve, and which had a mean latency of 21·4 ± 2·1 msec. All the units recorded on the ipsilateral side responded to stimulation of the A$\gamma\delta$ fibres. The mean latency of this group was 18·5 ± 1·7 msec.

4. A high proportion of the splanchnic units of both classes could be activated by stimulating the appropriate part of the body surface, and this indicates that the cutaneous and splanchnic pathways converge at or below the level of the thalamus.

5. Unit responses have also been recorded in the region of the posterior ventral nucleus of the thalamus of a baboon, following electrical stimulation of the A$\gamma\delta$ fibres in the splanchnic nerve of the opposite side. Some of these units were also activated by stimulating the appropriate part of the body surface.

6. The relation of these findings to the subject of referred pain is discussed.

I should like to express my thanks to Dr G. Gordon for his constant encouragement and advice and his helpful criticism throughout this work. I am indebted to Mr E. H. Leach and Mr T. A. Marsland for all the photomicrography.

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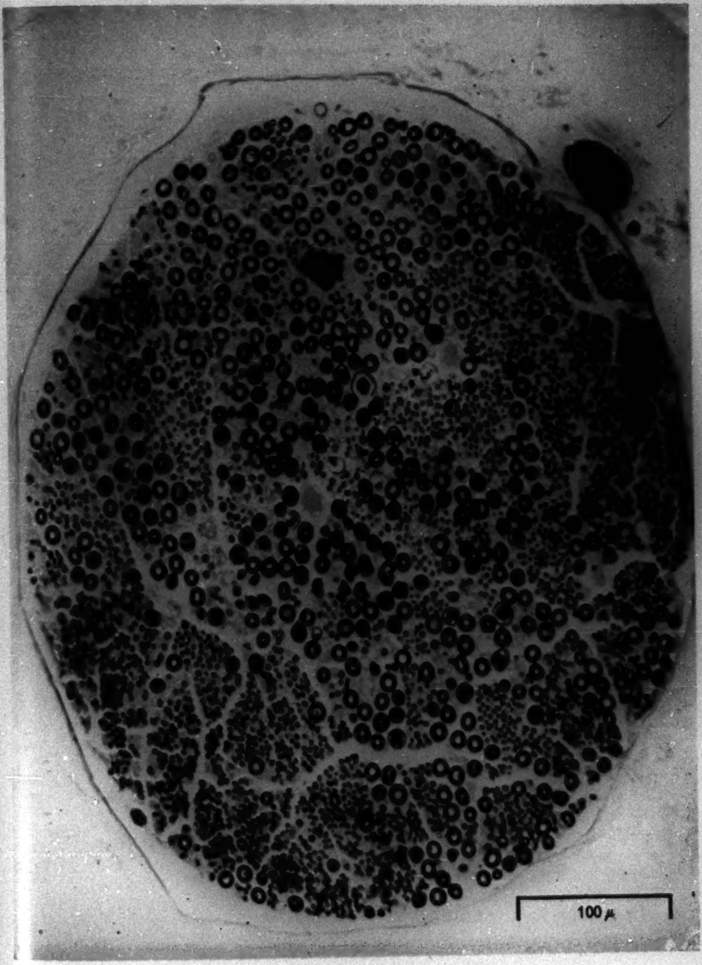
EXPLANATION OF PLATE

Plate 1. Photomicrograph of a cross-section of a cat's splanchnic nerve stained with osmic acid. The fibre spectrum of this nerve is shown in Fig. 3.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POTASSIUM MOVEMENTS AND ATP IN HUMAN RED CELLS

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It has been known since 1941 that human red cells depend upon glycolysis for the maintenance of concentration gradients of sodium and potassium across the cell membranes (Harris, 1941; Danowski, 1941). Glycolysis is also needed to maintain the concentrations of adenosine triphosphoric acid (ATP) and other phosphate esters, notably 2:3-diphosphoglyceric acid (2:3-PGA; see review by Prankerd, 1955), and ATP is sometimes regarded as the energy transmitter between glycolysis and the active transport of cations. To find if the ATP concentration and the active uptake of potassium were correlated in human red cells, the ATP and potassium concentrations have been measured in cells maintaining, losing and gaining potassium.

The effects of digoxin and ouabain (strophanthin-G), which inhibit the active movements of sodium and potassium without affecting glycolysis (Schatzmann, 1953), were also studied to find if they caused a depletion of intracellular ATP.

The results are consistent with the view that ATP is at least one link in the coupling between glycolysis and the active influx of potassium. The effects of cardiac glycosides, however, show that ATP is not the only link, and are interpreted as indirect evidence for the participation of a membrane carrier in the linked transport of potassium with sodium across the cell membrane. A short account of this work has been published (Whittam, 1957).

METHODS

Preparation of cells. Blood was drawn from the antecubital veins of healthy young men and collected in a flask containing heparin (Pularin, Evans Medical Supplies, 1 mg/10 ml. blood). It was immediately centrifuged and the plasma and buffy coat removed by suction. The cells were then suspended in four volumes of 0·9% (w/v) NaCl solution and allowed to stand for about 1 min before being spun down. After centrifugation the saline and the upper layer of cells were removed and the washing repeated twice more, as this procedure has been shown to eliminate the white cells almost completely (Glynn, 1956). The cell suspension was then free of reducing sugar as tested by Benedict's sugar reagent. The washed cells were finally mixed with saline solution for incubation at

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37° C or for storage at 4° C. When additions of various substances were made to the suspension, concentrated solutions were added to give the concentrations shown in the text.

Incubation of cells. The cell suspensions were shaken in air in stoppered conical flasks in a water-bath at 37° C either fresh, immediately after the cells had been washed, or after storage for five days at 4° C in flasks stoppered with a plug of cotton wool. The temperature of the water-bath at 37° C was controlled by a thermostat and did not vary by more than 0·1° C during the incubations. Samples of the cell suspension were removed for analysis after various times. Precautions to ensure sterile conditions were not taken during cold-storage or incubation at 37° C but the latter never exceeded 5 hr and it is unlikely that bacterial growth would vitiate the results during this period. Samples of the suspension were taken for a haematocrit determination at the beginning and end of each incubation, and in some experiments each time a sample was taken for potassium and phosphate analyses.

Medium. A saline solution buffered with phosphate to pH 7·5 was used and was similar to that of Krebs & Henseleit (1932) save that half of the $MgSO_4$ was replaced by $MgCl_2$. This is recommended by Krebs (1950) in order to make the medium more like human plasma, which contains less sulphate than the plasma of several laboratory animals (Guillaumin, 1940). The ionic composition of the medium was as follows (mM): Na^+, 150; K^+, 5·0; Mg^{2+}, 1·1; Cl^-, 137; SO_4^{2-}, 0·6; $H_2PO_4^-$, 1·4; HPO_4^{2-}, 8·6.

Haematocrit determinations were used as an index of changes in cell volume during an experiment. It was necessary to compare changes in potassium and phosphate esters in the same number of cells (see Maizels, 1943). All cell concentrations are therefore 'corrected' by reference to the initial cell volume, and are therefore expressed in m-mole/l. cells at the start of an incubation at 37° C or of cold-storage at 4° C.

The actual determinations were made by centrifuging the suspension in capillary tubing of uniform bore (13 cm long and 3 mm bore) for 30 min at 1500 *g*. No change in volume occurred when spinning was continued for a further 30 min. Since the volume of trapped fluid between the cells under these conditions is probably not more than 2% (Jackson & Nutt, 1951), the haematocrit value was taken to represent the actual cell volume. Corrections for haemolysed cells were not necessary, since lysis was never more than about 4%.

Analysis procedure

Each sample for analysis was centrifuged for 5 min at 1500 *g* and most of the supernatant fluid removed with a Pasteur pipette and its volume measured by placing it in a 5·0 ml. graduated tube. The volume of cells and saline solution left in the tube was found by difference. The cells (0·5–1·0 ml.) were haemolysed with 0·5 ml. water containing a little saponin; 0·2 ml. of 100% (w/v) trichloroacetic acid (TCA) was added to deproteinize, and the mixture then removed and well shaken and centrifuged. The clear TCA extract was removed and stored at −12° C until the analyses were made. No phosphate esters were found in the medium and a correction for medium in the TCA extract was therefore unnecessary since the esters therein must all have been derived from the cells. When orthophosphate or total acid soluble phosphates were analysed, however, a correction of the TCA extract values for the orthophosphate present in the trapped medium was made. This volume of medium was found by subtraction of the cell volume from the total volume of the suspension to which TCA was added. In the calculation of the volume of water in the TCA extract the cells were assumed to have a water content of 70% (w/v) (Maizels, 1943).

Haemoglobin was estimated as oxyhaemoglobin using a Spekker colorimeter, and an Ilford narrow-band yellow-green filter, to measure the optical density of the solutions. It was estimated in the medium after incubation and in the whole suspension. Lysis was determined by dividing the haemoglobin concentration in the medium by that in the whole suspension and multiplying the dividend by the fraction of the suspension occupied by the medium.

Potassium estimations were made with an EEL flame photometer either on suitable dilutions of the cell TCA extracts or on haemolysates of packed cells. The small amount of TCA (less than 2 mM) did not interfere with the readings, which were made in quadruplicate, alternating the unknown solutions between standard solutions. The results gave a S.E. of the mean of less than 2%.

Lactic acid estimations on TCA extracts of the whole cell suspension (4·0 ml. suspension and 1·0 ml. 5% (w/v) TCA) were made by the method of Barker & Summerson (1941), and pyruvic acid was estimated likewise by the method of Friedmann & Haugen (1943).

Total acid-soluble phosphate and orthophosphate in the TCA extracts were estimated by the method of Fiske & Subbarow (1925).

Phosphate esters in the TCA extracts were first separated by one-dimensional paper chromatography at room temperature using solvent no. 3 of Gerlach, Weber & Döring (1955). Whatman No. 1 filter paper was first washed with 0·2% (w/v) ethylenediamino-tetraacetic acid solution and water as described by Eggleston & Hems (1952) and a satisfactory separation of ATP, ADP, AMP, 2:3-PGA, orthophosphate, and hexose phosphates was obtained. After trial separations using the same solvent several times, when occasionally all the compounds moved with the orthophosphate, a fresh solvent solution was prepared each time before use. About 100 μl. of TCA extract was accurately applied to the base line of the chromatogram, 7·2 μl. at a time, using a graduated micropipette of a self-filling type similar in principle to that described by Kirk (1950). The paper was dried in a stream of cold air from a hair dryer before the addition of each sample.

After removal from the solvent the chromatograms were dried at room temperature and examined under a low-pressure Hg lamp (Chromatolite, Hanovia, Slough) when compounds containing purines absorbed ultra-violet light and were located as dark areas on a light background. Photographs of the chromatograms under ultra-violet light were also usually made, essentially as described by Markham & Smith (1949).

The chromatograms were sprayed with the molybdate solution of Hanes & Isherwood (1949) and the areas containing phosphates were developed as blue spots by exposing the paper to ultraviolet light (Bandurski & Axelrod, 1951) which was found to be much less troublesome than the use of a jar of H_2S for the reduction of the phospho-molybdate complex. The areas of paper containing the phosphates were ashed with a mixture of H_2SO_4 and $HClO_4$ (Hanes & Isherwood, 1949) and the orthophosphate estimated by the method of Berenblum & Chain (1936).

In order to check the identity of the compound from cell extracts which behaved as ATP on the chromatograms and which absorbed ultra-violet light, the area of paper was cut out before spraying with molybdate and allowed to stand during the night at 4° C in 2 ml. of water. The spectrum of the eluate in the ultra-violet between 240 and 270 mμ was then determined in a Uvispek spectrophotometer (Hilger and Watts Ltd., London). A single peak was found at 260 mμ which is characteristic of adenine compounds, and from the extinction coefficient (Kalckar, 1947) the concentration of purine was calculated. The phosphate in the same eluate was also estimated after acid hydrolysis and the ratio of purine to phosphate was approximately 3:1, which is to be expected for ATP.

Accuracy of phosphate analyses. Duplicate analyses of cells from the same suspension gave values which agreed within 10%.

Influx measurements of potassium were made using ^{42}K and following the procedure of Glynn (1956).

RESULTS

Phosphate compounds in human red cells

Values in the literature for the concentrations of ATP, ADP and 2:3-PGA in fresh, normal human red cells show wide variations which are independent of the analytical methods employed. Thus, values given by different authors for ATP concentrations have a range of four- or fivefold and even values from the same authors show a range of two- or threefold (Table 1). The differences in ATP and ADP concentrations found with the present analytical methods cannot be ascribed to variable losses of the compounds due to their instability because the same technique of handling different samples of cells was followed, and, moreover, almost identical values were found for cells from the same suspension (see Methods). The observations that red cells from subjects with acidosis contain less 2:3-PGA than normal cells (Rapoport & Guest, 1939) may explain some of the lower values from the wide range for the 2:3-PGA concentration, but there is still a considerable spread for cells from donors apparently healthy. From the values given in Table 1 it seems possible that wide differences may exist in phosphate ester concentrations in red cells from different individuals and from the same individual at different times (e.g. the ATP concentration in red cells obtained on different occasions from the same healthy individual varied from 0·8 to 1·2 m-mole/l. cells).

TABLE 1. Concentrations of phosphate esters in human red cells (m-mole/l. cells)

Analytical method	ATP	ADP	2:3-PGA	Reference
Chromatography	0·90 ± 0·08 0·5–1·2 (10)	0·48 ± 0·03 0·4–0·6 (5)	4·62 ± 0·24 3·3–5·4 (8)	This paper. The S.E. of the mean, the range and the number of observations are given
Chromatography	0·3–0·9	0·2–0·4	2·0–2·9	Prankerd & Altman (1954)
Chromatography	1·16	0·25	—	Overgaard-Hansen, Jørgenson & Praetorius (1957)
Chromatography	0·76 0·47–1·11	0·36 0·20–0·43	4·7 3·7–5·9	Fleckenstein & Gerlach (1953); mean values and range
Chromatography	1·0 0·7–1·4	None detected	2·1 1·8–3·3	Rhodewald & Weber (1956); mean values and range
Ion exchange resin	0·45	0·12	1·6	Bartlett, Savage, Hughes & Marlow (1953)
Differential hydrolysis	1·4 (7 min P)	—	4·5	Rapoport & Guest (1941)
Differential hydrolysis	1·1	None detected	3·7	Gourley (1952)
Differential hydrolysis	1·3 (7 min P)	—	5·0	Farmer & Maizels (1939)

In spite of the variation in results, a point clearly emerging from Table 1 is that human red cells contain at least two or three times as much ATP as ADP and that the 2:3-PGA concentration is about four times greater than the sum of the ATP and ADP concentrations. About 80% of the acid-soluble phosphate consisted of 2:3-PGA, ATP and ADP.

The variation in potassium concentrations in fresh red cells was small compared with that in phosphate concentrations. The mean potassium concentration from ten experiments was 98·9 ± 1·6 m-mole/l. cells (S.E. of the mean) with a range from 92 to 106. There was no correlation between the potassium and ATP concentrations, a high potassium concentration sometimes being found with a low ATP concentration and vice versa.

Incubation of fresh cells

Cells incubated in a saline solution containing glucose maintained the concentrations of ATP, ADP and potassium (Table 2), but after 5 hr a 30% fall had occurred in the 2:3-PGA concentration. No other organic phosphate was detected (e.g. 2- or 3-phosphoglyceric acid or phospho-enol pyruvate), but the inorganic phosphate concentration increased, indicating hydrolysis of the 2:3-PGA. The initial glucose concentration was 11 mM, which represents a ninefold excess over that required for a typical consumption of 1·5 m-mole/l. cells/hr (Maizels, 1951; Bernstein, 1953), and it is not clear why the cells should have utilized their store of 2:3-PGA in a buffered suspension when glucose and ATP were still available to facilitate glycolysis.

Table 2. Incubation of fresh cells at 37° C. Concentrations of phosphates and of K are shown (m-mole/l. cells) before and after incubation in the presence and absence of glucose

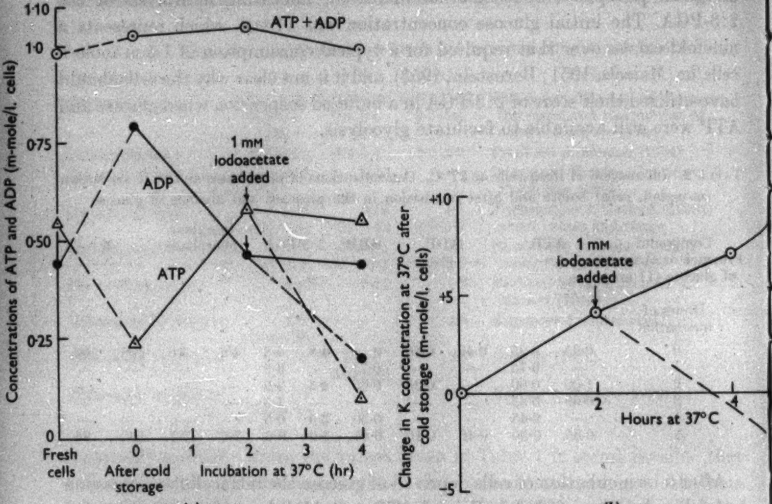
Compound ...	ATP		ADP		AMP	2:3-PGA		Inorganic phosphate		K	
Presence or absence of glucose (11 mM)...	+	−	+	−	−	+	−	+	−	+	−
Hours of incubation											
0	0·95	0·95	0·40	0·40	0	4·8	4·8	4·6	4·6	106	106
1	—	0·75	—	—	0·75	—	3·5	—	—	—	—
2	1·00	0·60	—	0·25	0·65	4·5	2·0	—	—	—	—
3	0·95	0·55	—	—	—	—	1·5	—	—	—	—
4	—	0·45	—	—	0·50	3·5	0·5	—	—	—	—
5	0·85	0·30	0·40	0·10	0·45	3·0	0·5	7·6	13·7	105	96

After 5 hr incubation of cells deprived of glucose the intracellular potassium had fallen by 10 m-mole/l. cells and ATP and ADP by 60%. 2:3-PGA was almost completely hydrolysed with a corresponding rise in inorganic phosphate (Table 2). A similar result was found in cells incubated in the presence of 1 mM-iodoacetate (IAA). About 0·5 m-mole AMP/l. cells was produced but this is 0·45 m-mole/l. cells too little to balance the loss of ATP and the deficit in nucleotide after 5 hr may be due to the formation of small amounts of hypoxanthine or adenine.

Incubation of cold-stored cells

Cells cold-stored with glucose. During incubation of fresh cells at 37° C the potassium concentration either remained constant or decreased, so that in order to compare phosphate changes with a net uptake of potassium, cells first had to be stored at 4° C in order to lower their potassium, and then incubated at 37° C. It was desirable to arrange the time of storage at 4° C so that a measurable net accumulation of potassium could be observed during 5 hr incubation with glucose at 37° C; Ponder (1949) found most potassium uptake after cold storage for 5 days and this period of storage was therefore chosen in the present experiments. The medium contained glucose during both cold storage and the subsequent incubation at 37° C.

Potassium leaked out of the cells at a very low rate (about 0·1–0·2 m-mole/l. cells/hr) during cold storage, and since ATP was still present this loss was probably due to a direct effect of the low temperature on the mechanism for potassium transport.



(*a*) (*b*)

Fig. 1. Changes in ATP and ADP (*a*) and in K (*b*) during storage of human red cells at 4° C and on subsequent incubation at 37° C. The arrows indicate the addition of IAA at 37° C and the broken lines at 37° C show the changes in the IAA-treated cells.

Incubation of cold-stored cells

Potassium was accumulated by the cold-stored cells at 37° C at about 2 m-mole/l. cells/hr (Fig. 1*b*). This uptake was abolished by the addition of IAA (1 mM), which caused a loss of potassium, but, as Taylor, Weller & Hastings (1952) also found, the uptake of potassium was unaffected by physostigmine sulphate (0·5 mM) which was added as an inhibitor of ACh esterase to test if this enzyme was required for potassium uptake.

During 5 days cold storage about 60 % of the initial ATP was hydrolysed to ADP, but on incubation at 37° C the ADP thus formed was re-phosphorylated to ATP, which then remained constant (Fig. 1*a*). The concentration of 2:3-PGA remained constant during cold storage with glucose but fell about 40 % during incubation at 37° C.

Fig. 1 shows that potassium was accumulated at about the same rate during the whole of the 4 hr incubation and that the ATP concentration increased during the first 2 hr and then remained constant. (Intermediate values during the first 2 hr were not obtained because the small changes in the potassium concentration would have been within experimental error.) The result obtained might suggest that once ATP was available, both during and after its resynthesis, the rate of uptake of potassium was not correlated with the ATP concentration. This conclusion would be premature, however, because if the ATP concentration increased during the first few minutes to its 2 hr level the rate of uptake would still correlate with the ATP concentration. Since the measurement of net changes in potassium concentration is too insensitive to distinguish between these possibilities, the simultaneous measurement of potassium influx (with tracer potassium) and ATP concentration at frequent intervals during the first 2 hr of incubation is required.

Fate of 2:3-*PGA*

The loss of 2:3-PGA during the incubation of fresh cells at 37° C showed that it was utilized much more when glucose was absent than when this was present and the question arises as to the end product of its metabolism. Estimations of lactic and pyruvic acids were therefore made on cell suspensions to test if these acids were the products. No lactic acid was detected in a glucose-free suspension whilst cells containing glucose produced about 3·9 m-mole lactic acid/l. cells/hr. Fig. 2 shows the loss of 2:3-PGA and production of pyruvic acid

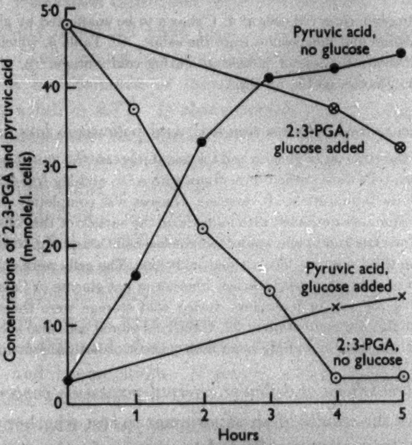


Fig. 2. Conversion of 2:3-PGA to pyruvic acid during the incubation at 37° C of fresh red cells in the presence and absence of glucose.

during 5 hr incubation of cells in the presence and in the absence of glucose. In each condition 2:3-PGA was quantitatively converted to pyruvic acid, the rate in the glucose-free suspension during the first 2 hr being 1·5 m-mole/l. cells/hr.

Pyruvic acid is to be expected because in the absence of glucose no co-enzyme 1 will be reduced by triosephosphate dehydrogenase and therefore lactic acid dehydrogenase, lacking its co-enzyme, will be unable to convert pyruvic acid to lactic acid. In accord with this result, Pappius & Denstedt (1954) have observed an accumulation of pyruvic acid and a decrease in 'difficultly-hydrolysable' phosphate (probably 2:3-PGA) in blood cold-stored without glucose.

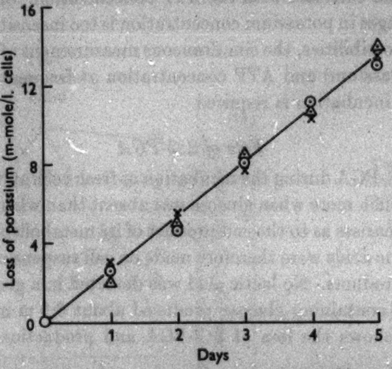


Fig. 3. Loss of potassium from red cells at 4° C shown to be unaffected by glucose deprivation or removal of calcium and magnesium from the saline. Cf. Table 5, which shows that digoxin also did not increase the loss of potassium during cold storage. ⊙, glucose added; ×, no glucose; △, no glucose, no Ca, no Mg.

Glucose and calcium removal, and potassium loss at 4° C

To test whether the removal of glucose and Ca would increase the loss of potassium during cold storage at 4° C, fresh cells were washed three times with a Ca- and Mg-free saline containing 0·2% (w/v) ethylenediamine tetraacetic acid (versene). Glucose was completely washed out of the cells and the versene presumably combined with ionic Ca on the outside of the cells. The cell suspension was divided into three lots and to one was added glucose and Ca and Mg, to another was added Ca and Mg, whilst the third one was left without additions. The cells were cold-stored, and Fig. 3 shows that the loss of potassium was identical whether or not glucose or Ca and Mg were present. The factors limiting the loss of potassium during cold storage were therefore independent of glucose and Ca and Mg. On incubation at 37° C the cold-stored cells also accumulated potassium to the same extent whether Ca and Mg were both present or both absent in the saline.

ATP breakdown at different external potassium concentrations

Table 3 shows the results of an experiment to test whether the hydrolysis of ATP in glucose-free cells was affected by changes in the external sodium and potassium concentrations, which are known to change critically the fluxes of these ions (Glynn, 1956). A cell suspension was prepared with the saline potassium concentration equal to 0, 5 and 100 mM, the balance for isotonicity being made up with sodium chloride. The suspension had a low haematocrit of 5% so that changes in medium potassium with an initial zero concentration, due to leakages from the cells, would be negligible. It might be expected that at zero and 100 mM external potassium concentrations the ATP hydrolysis would be retarded, since the linked active potassium uptake and sodium extrusion is abolished with zero external potassium (Glynn, 1956), and with 100 mM-K the concentration gradients of sodium and potassium between cells and saline are much reduced. However, the results show that the break-down of ATP and 2:3-PGA proceeded at the same rate at the three different potassium concentrations (Table 3). Clearly no sparing effect on ATP break-down was elicited by the changes in sodium and potassium composition of the saline solution.

TABLE 3. Break-down of ATP and 2:3-PGA in glucose-free red cells in saline with different Na and K concentrations (m-mole/l. cells)

Compound ...	ATP			2:3-PGA		
Na concn. (mM) ...	150	145	50	150	145	50
K concn. (mM) ...	0	5	100	0	5	100
Hours of incubation						
0	0·80	0·80	0·80	5·00	5·00	5·00
1	0·75	0·70	0·75	4·65	4·75	4·40
2	0·70	0·65	0·60	3·45	3·60	3·30
3	0·55	0·45	0·50	2·35	2·25	2·00

Potassium influx and ATP concentration

The results already described have been of net movements of potassium either into or out of cells, and it has been shown that a potassium uptake was accompanied by a maintenance or increase in ATP concentration and a potassium loss by a fall in ATP. It also emerged that 2:3-PGA almost disappeared from a glucose-free suspension, owing to conversion to pyruvic acid, which suggests that 2:3-PGA, as a reserve source of energy, may be capable of maintaining potassium influx in the absence of glucose. To test whether potassium influx was maintained in the absence of glucose, and whether its decline was correlated with a fall in ATP, the following experiment was performed.

A washed glucose-free suspension of cells was divided into five flasks and ^{42}KCl was added to them at intervals of 1 hr. To measure the potassium influx as a function of time, samples were removed from the flasks at 30 and 60 min after the addition of ^{42}K, and after centrifugation the cells were washed and analysed for ^{42}K and haemoglobin. A graph of activity against time was drawn for each flask and from the slopes of the straight lines obtained the influx at each hourly interval was calculated. Measurements of ATP and 2:3-PGA were also made on cells taken at hourly intervals. The same measurements were also made on cells incubated in the presence of glucose. The results

(Fig. 4) show that, as expected, the potassium influx and ATP concentration in cells containing glucose were the same after 4 hr incubation as initially. The initial influx was 2·0 m-mole/l. cells/hr and the initial ATP concentration was 1·2 m-mole/l. cells. The potassium influx into glucose-deprived cells, on the other hand, was reduced by 15% even after 1 hr incubation and the fall continued, until after 4 hr incubation the influx was but 57% of its initial value. When expressed as a percentage of the initial value, the ATP concentration fell in almost the same way as the potassium influx. 2:3-PGA was utilized in a way similar to that shown in Fig. 3. The rate of decline in potassium influx and ATP concentration was thus very close, and Fig. 4 shows that in spite of its conversion to pyruvate, 2:3-PGA was unable to maintain the potassium influx and ATP concentration at their initial levels.

Ratio of potassium influx to ATP hydrolysis

From the work of Rapoport & Luebering (1952), which demonstrated the conversion of 2:3-PGA to 3-PGA during its metabolism to pyruvic acid, it is reasonable to assume that only one molecule of ATP is formed per molecule of

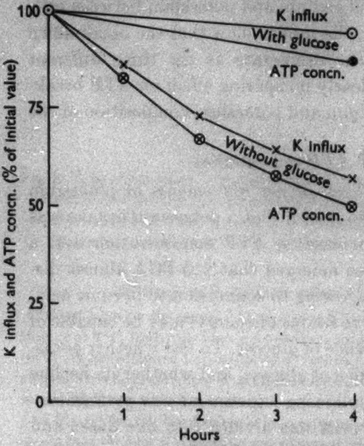


Fig. 4

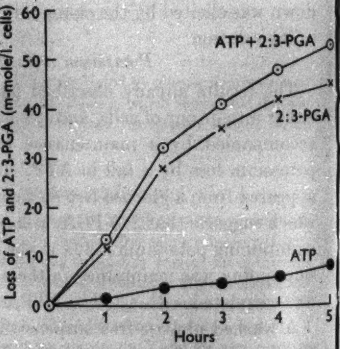


Fig. 5

Fig. 4. Change in potassium influx and ATP concentration in human red cells in the presence and absence of glucose. Results are plotted as percentages of the initial values. Potassium influx values were obtained by measuring the rate of uptake of ^{42}K, which was added to separate suspensions of cells at hourly intervals.

Fig. 5. The total loss of ATP and 2:3-PGA from cells deprived of glucose during incubation at 37° C. The total loss of the two compounds (◎) is given as a measure of the total hydrolysis of ATP, assuming that one molecule of ATP was synthesized per molecule of 2:3-PGA metabolized.

2:3-PGA utilized. The rate of disappearance of 2:3-PGA from the cells was measured (see Fig. 3) and has accordingly been taken to represent the rate of ATP synthesis due to 2:3-PGA metabolism. Since the intracellular ATP concentration showed a progressive fall (Fig. 4) the total rate of hydrolysis of ATP must have been equal to the rate of synthesis from 2:3-PGA plus the rate of loss of endogenous ATP. The total loss of ATP computed in this way is shown in Fig. 5. The amounts of ATP hydrolysed and the mean values for potassium influx during each of the first 4 hr of incubation are given in Table 4. The ratio of potassium transported inwards to ATP hydrolysed was about 1·4. It is worth emphasizing that this ratio is derived solely from measurements of potassium influx and the loss of 2:3-PGA and ATP in cells deprived of glucose, and the only assumptions made are that one molecule of ATP was synthesized during the conversion of 2:3-PGA to pyruvic acid and that all the intracellular ATP was available for the transport mechanism. The similar results of a duplicate experiment (number 2) are also shown in Table 4.

TABLE 4. Comparison of K influx and ATP hydrolysis in glucose-deprived cells

	ATP hydrolysed (m-mole/l. cells)	Mean K influx (m-mole/l. cells)	$\frac{\text{Mean K influx}}{\text{ATP hydrolysed}}$
Experiment 1			
1st hour	1·37	1·93	1·4
2nd hour	1·41	1·64	1·2
3rd hour	1·03	1·42	1·4
4th hour	0·83	1·26	1·5
Experiment 2			
1st hour	1·35	2·00	1·5
2nd hour	1·45	1·73	1·2
3rd hour	1·15	1·51	1·3
4th hour	1·00	1·29	1·3

If ATP is indeed the source of the energy for the linked active transport of sodium and potassium, the fraction of the free energy of hydrolysis of ATP utilized in this way can be calculated. For a linked transport of sodium and potassium the free energy required for the movement of one equivalent of cation is

$$RT \ln \frac{[\mathrm{K}]_i [\mathrm{Na}]_o}{[\mathrm{K}]_o [\mathrm{Na}]_i},$$

where the subscripts refer to the internal (i) and external (o) concentrations—concentrations here correspond respectively to m-mole/l. water in cells or external medium—and other terms have their usual significance. Substitution of values for the internal and external cation concentrations and the influx value for the first hour, gives the energy requirement as 3030 cal. The standard free energy of hydrolysis of the terminal phosphate group of ATP is about 5600 cal (Vladimirov, Vlassova, Kolotilova, Lyzlova & Panteleyeva, 1957) and thus the percentage of the observed hydrolysis energy required for the cation transport is 75% $\left(=\frac{1\cdot4\times3030}{5600}\right)$. The available ATP was thus sufficient to satisfy the energy requirement of the cation transport, even on the disadvantageous assumption that the whole of the potassium influx and sodium efflux were active.

Effect of digoxin

Schatzmann showed in 1953 that digoxin and other cardiac glycosides prevented the uptake of potassium at 37° C by cold-stored human red cells which otherwise occurred in the presence of glucose, and moreover, that this effect was not due to an inhibition of glycolysis. More recent work on the kinetics of sodium and potassium movements in the presence of digoxin has shown that the active fluxes of these ions are reduced at least to the values found in cells deprived of glucose (Kahn & Acheson, 1955; Joyce & Weatherall, 1955; Glynn, 1955, 1957). It therefore seemed worth testing whether digoxin might act by interfering with the supply of ATP from glycolysis. To test this possibility incubations were made at 37° C with both fresh and cold-stored cells both in the presence and absence of glucose.

Freshly washed cells were suspended in two lots of medium, identical save that glucose was added to one and not to the other. Each sample was further divided into two lots, to one of which digoxin was added to give a final concentration of 0·01 mM. This arrangement was designed to show whether digoxin caused a greater loss of potassium than glucose deprivation alone. Within the limits of experimental error, the results of incubation at 37° C (Table 5) show that digoxin in the presence of glucose caused only the same net loss of potassium (2 m-mole/l. cells/hr) as that from cells deprived of glucose, and also that the addition of digoxin did not increase the loss of potassium from glucose-deprived cells. The potassium concentration was maintained in the control cells without digoxin. In contrast to the loss of potassium with digoxin in the presence of glucose, the concentrations of ATP, ADP, 2:3-PGA and inorganic phosphate remained the same in cells incubated both with and without digoxin.

In a glucose-free suspension, the addition of digoxin (or of ouabain) markedly retarded the hydrolysis of ATP during the first 2 hr of incubation (Fig. 6*a*). In two other experiments of 1 hr duration the inhibition of dephosphorylation of ATP was 79 and 94%. This sparing effect was observed only during the first 2 hr and the rate of hydrolysis after then was about the same as that in cells without the glycoside. Fig. 6*a* shows that the utilization of 2:3-PGA in glucose-free cells was unaffected by digoxin or ouabain. Fig. 6*b* shows the total loss of ATP and of 2:3-PGA.

Table 5 shows the results of cold storage of cells with and without digoxin in the presence and absence of glucose. During cold storage, the same amount of potassium was lost under each of the four conditions and this shows, first, that

TABLE 5. Incubation of fresh and cold-stored red cells in the presence and absence of digoxin (10^{-5} M) and glucose (11 mM). Cells were incubated for 5 hr at 37° C either fresh or after cold storage at 4° C for 5 days. Concentrations are given in m-mole/l. cells

				Inorganic phosphate			
Compound	ATP	ADP	2:3-PGA	Cells	External saline medium	Total acid-soluble phosphate	K
Fresh cells							
Before incubation	0·95	0·40	5·5	4·0	10·0	19·0	104
After incubation with glucose	0·85	0·40	3·0	7·5	7·5	17·0	103
with glucose and digoxin	0·90	0·50	3·5	7·0	7·5	17·5	94
without glucose	0·60	0·40	1·5	13·5	10·0	20·0	95
without glucose, with digoxin	0·60	0·40	1·5	14·0	10·0	20·0	94
Cold-stored cells							
After storage with glucose	0·55	0·50	5·5	5·5	8·5	19·5	84
After incubation at 37° C	0·70	0·50	1·5	11·0	10·5	17·5	88
After storage with glucose and digoxin	0·50	0·45	5·5	6·5	8·5	19·5	83
After incubation at 37° C	0·65	—	2·0	12·5	8·5	19·0	76
After storage without glucose	0·70	0·70	0·5	15·5	9·0	21·0	84
After incubation at 37° C	0	0·40	0	14·5	9·5	18·5	78
After storage without glucose, with digoxin	0·60	0·70	1·0	16·0	9·0	20·5	86
After incubation at 37° C	0	0·35	0	13·0	10·5	16·0	79

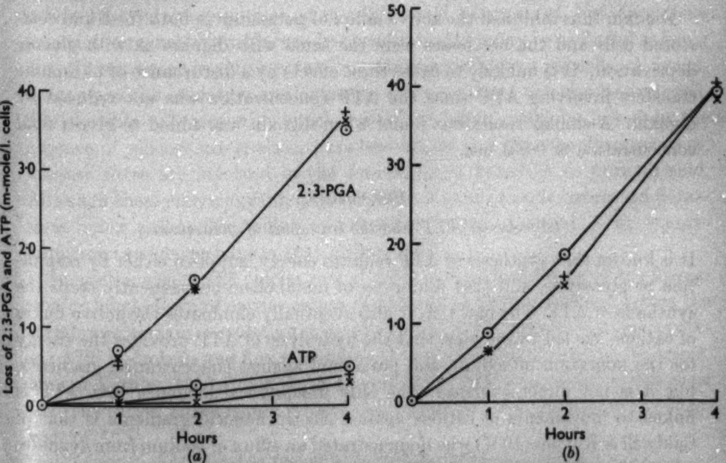


Fig. 6. The effect of ouabain (10^{-6} M) and digoxin (10^{-5} M) on ATP hydrolysis in red cells deprived of glucose during incubation at 37° C. The loss of 2:3-PGA was unaffected by the glycosides but the values are plotted (*a*) separately and (*b*) after addition to the ATP values in order to show the total hydrolysis of ATP and the initial retardation effect of ouabain and digoxin. ⊙, cells without glycoside added; ×, 10^{-6} M ouabain added; +, 10^{-5} M digoxin added.

glucose deprivation did not increase the loss of potassium after 5 days storage (see also Fig. 3, and Maizels, 1949), and secondly, that the addition of digoxin did not increase the loss of potassium at 4° C either in the presence or absence of glucose.

After cold storage with glucose the same concentrations of the different phosphate fractions were found in cells stored with and without digoxin. Thus, the ATP fell during cold storage whilst the other phosphates did not change markedly. In agreement with Schatzmann (1953), it was found that digoxin prevented the recovery of potassium on 5 hr incubation at 37° C which occurred with glucose (4 m-mole K/l. cells), and, instead, a loss of 7 m-mole K/l. cells was observed. The phosphates of the various fractions were again unaffected by digoxin in the presence of glucose (Table 5) and a small increase in ATP plus ADP, a large fall in other organic phosphates and a rise in orthophosphate are shown by the values.

In the absence of glucose the cells lost the same amount of potassium (6–7 m-mole/l. cells) at 37° C in suspensions both with and without digoxin, and this loss was the same as that found in the cells with glucose and digoxin. After cold storage without glucose most of the 2:3-PGA had disappeared, with a corresponding rise in inorganic phosphate, and after 5 hr at 37° C the cells had lost about 80% of ATP plus ADP. The endogenous store of 2:3-PGA was thus utilized during cold storage when, evidently, the cells were not completely metabolically inert.

Digoxin thus inhibited the active influx of potassium in both fresh and cold-stored cells and the net losses were the same with digoxin as with glucose deprivation. It is unlikely to cause these effects by a disturbance of phosphate transfers involving ATP since the ATP concentration was not reduced by digoxin. A similar result was found when digoxin was added to give a final concentration of 0·001 mM.

DISCUSSION

Glycolysis, ATP and the transport of potassium

It is known that synthesis of ATP requires energy, supplied either by respiration or glycolysis, and that inhibition of metabolism consequently stops the synthesis of ATP. The fact that it also eventually eliminates the active fluxes of cations has led to the view that the hydrolysis of ATP provides the energy for the migration of sodium and potassium against concentration gradients, but it is not direct evidence. The only unequivocal evidence that ATP is linked to movements of cations against electrochemical gradients is that of Caldwell & Keynes (1957) who demonstrated an efflux of sodium from cyanide-poisoned nerve axons when ATP was injected. Support for a similar linkage in red cells is given by Prankerd (1956) and in this paper.

Human erythrocytes in which the potassium concentrations were rising, falling and being maintained constant have been analysed for phosphate esters and potassium, and the main finding is that ATP was always present when the intracellular potassium was rising or being maintained, whilst a fall in ATP occurred when potassium was leaking into the medium. Thus, during the incubation of fresh cells with glucose at 37° C both potassium and ATP were maintained constant, and during similar incubation after cold storage with glucose a rise in the intracellular concentrations of both ATP and potassium were observed. Conversely, when the potassium concentration was falling, as during incubation with IAA or in the absence of glucose, a fall of ATP and of potassium occurred. Gerlach (1956) and Prankerd & Altman (1954) have made similar observations on the phosphate compounds. These results are only compatible with, and are not direct evidence for, the ATP hypothesis, since the same results would be obtained if the active transport of potassium and the synthesis of ATP depended separately upon glycolysis without the transport of potassium being directly dependent upon ATP.

The most suggestive indication of a causal linkage between active potassium influx and ATP, and evidence against a dependence of influx upon the whole of the reactions of glycolysis, are provided by the observation that in cells producing not lactic acid but pyruvic acid the rates of fall in influx and ATP concentration were very similar (Table 4). This correlation suggests that in the absence of glucose ATP prevented an abrupt fall in potassium influx to its final low level. If the influx had been coupled to a glycolytic enzyme at a point before phosphoglyceromutase, such a fall would be expected, but since these enzymes were inactive in glucose-free conditions they may be excluded as being directly concerned in active potassium transport in human red cells. A dependence upon the oxidation and reduction of co-enzyme 1 can also be excluded. The possible enzymes with which potassium influx could be linked in the absence of glucose are enolase, pyruvate kinase and phosphoglyceromutase, because these are involved in the conversion of 2:3-PGA to pyruvic acid. Although these enzymes cannot be excluded it seems simpler to regard potassium influx and sodium efflux as being due to the operation of an energy-requiring carrier mechanism that utilizes ATP.

Suggestions as to the precise role of ATP such as facilitation of the formation or break-down of carrier-cation complexes, or their movement across the membrane, remain speculative at the present time. Energy calculations involving ATP hydrolysis and flux measurements can easily be misleading (Gillespie, Maw & Vernon, 1953; Whittam & Davies, 1954), but the present results show that the cation fluxes would require only about 75% of the energy of hydrolysis of ATP.

The role of 2:3-*PGA in red cells*

The role of 2:3-PGA in mammalian red cells is still obscure, for its conversion to pyruvic acid—ineffective as regards the complete maintenance of potassium influx—can hardly represent its physiological function. The present results agree with earlier ones in indicating the dynamic nature of 2:3-PGA and its close connexion with glycolysis (see Rapoport & Guest, 1939; also review by Guest & Rapoport, 1941). Farmer & Maizels (1939) suggested that the base-binding power of 2:3-PGA, other phosphorylated compounds and glutathione in the red cell may be equal to that of haemoglobin. Moreover, with the suitable pK value of 6·78 (Rubin, 1938), 2:3-PGA could participate in the acid-base buffering action of the blood in a way that intimately links this important physiological function with red cell metabolism.

ATP and 2:3-*PGA*

Rapoport & Luebering (1951) showed that human red cells contain an enzyme, glycerate-2:3-diphosphatase, which catalyses the hydrolysis of 2:3-PGA to 3-PGA. 3-PGA can be converted to phospho-enolpyruvate, which reacts with ADP to form ATP, and hence one molecule of ATP is formed per molecule of 2:3-PGA. Prankerd & Altman (1954), however, suggested that 2:3-PGA could give rise to two molecules of ATP by first undergoing a transformation to 1:3-PGA. This mechanism assumes the unlikely conversion of a secondary alcohol phosphate (2:3-PGA) into an acyl phosphate (1:3-PGA) and, furthermore, Rapoport & Luebering (1952) have shown that the diphosphoglycerate mutase reaction is practically irreversible and proceeds only in the direction of 1:3-PGA to 2:3-PGA. It is likely therefore that the catabolism of 2:3-PGA provides the cell with only one molecule of ATP at the reaction catalysed by pyruvate kinase (Solvonuk & Collier, 1955).

Red cells and digoxin

Schatzmann (1953) showed that the accumulation of potassium by red cells was prevented by digoxin, although the glycolysis was unaffected, and further work on the kinetics of the sodium and potassium movements has shown that the active fluxes were abolished (Joyce & Weatherall, 1955; Kahn & Acheson, 1955; Solomon, Gill & Gold, 1956; Glynn, 1957). The results of the phosphate analyses show clearly that the effect of digoxin on the transport of potassium in the presence of glucose was not due to a depletion of intracellular ATP, and the effect may be due to the inactivation of a membrane carrier directly.

The sparing effect on ATP hydrolysis found in glucose-free cells (as also found by Dunham, 1957), in the presence of ouabain and digoxin, is added support for the carrier-ATP hypothesis for sodium and potassium transport, for the retardation in ATP hydrolysis by an agent known to eliminate active fluxes of sodium and potassium strongly suggests that the active fluxes normally utilize ATP.

SUMMARY

1. Measurements of ATP, ADP and 2:3-diphosphoglyceric acid (2:3-PGA) have been made in human red cells maintaining, increasing and losing their intracellular potassium.

2. Concentrations in human red cells (in m-mole/l. cells with S.E. of the mean) were: ATP, 0·90 ± 0·08; ADP, 0·48 ± 0·03; 2:3-PGA, 4·62 ± 0·24 and K, 98·9 ± 1·6. Fresh cells incubated in the presence of glucose maintained their initial phosphate and potassium concentrations, whilst in the absence of glucose potassium leaked out, ATP and 2:3-PGA were hydrolysed and adenylic acid accumulated. The 2:3-PGA hydrolysed was quantitatively converted to pyruvic acid. The hydrolysis of ATP and 2:3-PGA in glucose-free cells was unaffected when the external potassium concentrations were altered so as to reduce the concentration gradients.

3. During cold storage at 4° C ATP was partly hydrolysed to ADP and the potassium concentration fell; these changes were reversed on subsequent incubation with glucose at 37° C. Potassium leakage at 4° C was unaffected by removal of glucose and of Ca and Mg from the saline solution.

4. The rates of fall in potassium influx and ATP concentrations in glucose-free cells were similar, suggesting that potassium influx was facilitated by the presence of ATP.

5. Digoxin and ouabain caused a net loss of potassium from fresh red cells containing glucose but did not reduce the ATP concentration, whilst after cold storage the uptake of potassium on incubation at 37° C was prevented but not the resynthesis of ATP. In glucose-free cells digoxin and ouabain caused an initial delay in the hydrolysis of ATP.

6. The conclusion has been reached that ATP is at least one of the links in the coupling between glycolysis and the active transport of potassium in human red cells and that a membrane carrier, inactivated by digoxin and ouabain, utilizes ATP in the transport of potassium across the membrane.

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THE ACETYLCHOLINE SYSTEM OF THE ELECTRIC ORGAN OF *MALAPTERURUS ELECTRICUS*

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The suggestion that the electric organ of *Malapterurus electricus*, the electric catfish, has evolved phylogenetically from glandular elements of the epidermis (Fritsch, 1887) had been accepted (Bridge, 1904; Schnakenbeck, 1955; Nachmansohn, 1955) with only few exceptions (Ballowitz, 1899; Maurer, 1913; Romer, 1955). Recently, however, it was shown (Johnels, 1956) that this organ is derived from striated muscle as is the electric organ of all other fishes known in this respect (*Electrophorus electricus*, the electric eel, and the *Torpedo* species, the electric rays). The electroplates of the electric organ of *Malapterurus* differ from those of the other electric fishes in not following Pacini's Law: that means the innervated face of the electroplates of *Malapterurus* become positive instead of negative during the discharge.

In the present experiment the content of acetylcholine (ACh) and of true cholinesterase or acetylcholinesterase (AChE) of the electric organ of the *Malapterurus* has been examined. Nachmansohn (1955) postulates that the ACh–AChE system is responsible for the permeability changes associated with the generation of bio-electrical potentials, and according to Nachmansohn, Coates & Rothenberg (1946) there is a direct proportionality between voltage, number of plates per centimetre and AChE concentration of the electric organ. According to the view of Nachmansohn the electric organ of *Malapterurus* should contain ACh and AChE in about the same high concentration as the electric organ of the *Electrophorus* and of the *Torpedo* because the voltage delivered by the electric organs of these three species is of the same order. The following results, however, show that this is not the case.

METHODS

The electric organ of *Malapterurus* was dissected out from living specimens and immediately homogenized either in bicarbonate buffer for determining AChE, or in trichloroacetic acid for determining ACh. AChE activity was measured with the Warburg technique (Augustinsson, 1957) with acetylcholine chloride as substrate in optimal concentration (3×10^{-3} M). ACh was isolated and assayed according to the technique recently described by Augustinsson & Grahn (1954). The identity of the active constituent with ACh was established biologically, chromatographically and biochemically. For comparison, the AChE and ACh content was determined in some electric organs of the *Torpedo marmorata* (kindly supplied to us by the Stazione Zoologica in Naples) and the enzyme activity was determined as well in the electric organ of the *Raja batis* which was caught off the west coast of Sweden.

RESULTS

The results are given in Table 1, together with previous results of other authors. The AChE activity of the electric organ of the *Malapterurus* is approximately one-hundredth of that activity of *Electrophorus* and *Torpedo* and of the same magnitude as the activity of mammalian nerve fibres. The nerve to the electric organ of the *Malapterurus* also had about the same AChE activity (7 mg of ACh/hr/g). The enzyme present in the *Malapterurus* electric organ had the characteristic properties of acetylcholinesterase, e.g. the activity substrate concentration relationship.

TABLE 1. AChE activity and ACh content of various electric organs. Numbers in italics obtained in the present study; other values from the literature: *Augustinsson (1955); † Nachmansohn (1955); ‡ Feldberg & Fessard (1942); § Sawaya & Mendes (1951)

Species	Voltage delivered (V)	AChE activity (mg of ACh/hr/g tissue)	ACh content (μg/g tissue)
Malapterurus electricus	100–400*	*10–20*	*<0·5*
Electrophorus electricus	400–600*	2000–4000†	—
Torpedo marmorata	100–200*	*5000–7000*	*40–60*
		2000–3000†	50–100‡
Narcine brasiliensis	14–30*	800§	—
Raja batis	0·5*	*5–10*	—

The high AChE activity obtained with the electric organ of the *Torpedo* confirms previous results (Nachmansohn, 1940; Nachmansohn & Meyerhof, 1941). It is known that the activity varies considerably with the condition of the material (Augustinsson, 1955), but in the present experiments, in which various types of material were used, such as fresh or dried organs prepared from dead or living rays of various ages, the activity was always found to be as high as that found for the electric organ of the *Electrophorus*. The comparatively low AChE activity of the rudimentary electric organ of *Raja* also confirms previous observations.

The ACh content of the electric organ of the *Torpedo* was found to be 40–60 μg/g tissue. The same amount was found by Feldberg & Fessard (1942). The results obtained with the *Malapterurus* electric organ showed that this organ contained minute amounts of ACh, less than one-hundredth of the amount found in the organ of the *Torpedo*.

DISCUSSION

The results obtained thus demonstrate that the AChE activity and ACh content of the electric organ of the *Malapterurus* is much lower than in the electric organ of *Electrophorus* and the *Torpedo*, but the voltage produced by these three organs is of the same order of magnitude and the phylogenetic origin is also the same. It therefore seems to us that the ACh–AChE system cannot play the same significant role for the generation of electric potentials in the electric organ of the *Malapterurus* as it is said to play in the electric organs of the other species. Our results further stress the necessity of including the electric organ of the *Malapterurus*, which like that of the *Electrophorus* and the *Torpedo* is evolved phylogenetically from striated muscles, in studies which are intended to elucidate the processes underlying the generation of bio-electric potentials.

SUMMARY

1. The electric organ of *Malapterurus electricus* has been analysed for its content of acetylcholine and acetylcholinesterase.

2. That content is much lower in *Malapterurus* than in *Electrophorus* and *Torpedo*, although the voltage produced by all is of the same order of magnitude and the phylogenetic origin is the same.

3. The ACh–AChE system cannot play the same significant role for the generation of electric potentials in *Malapterurus* that is claimed for it in other species.

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PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

NATIONAL INSTITUTE FOR MEDICAL RESEARCH, THE RIDGEWAY, MILL HILL, LONDON, N.W.7

[illegible] November [illegible]

The simultaneous determination of both oxygen consumption and contractile response of an isolated tissue preparation employing a system for the continuous circulation of oxygen through the medium. [illegible]

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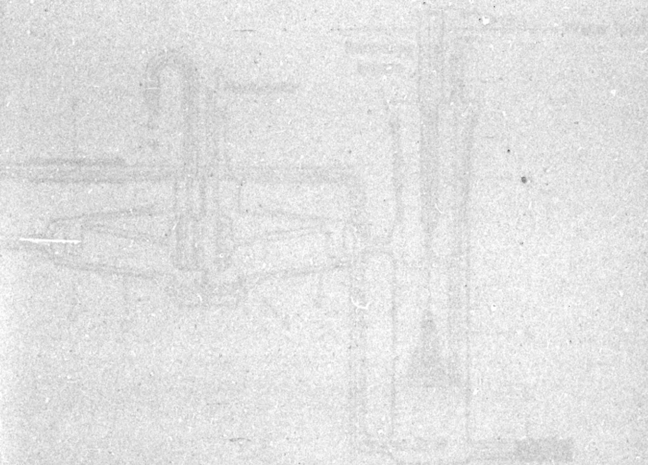

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PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

NATIONAL INSTITUTE FOR MEDICAL RESEARCH, THE RIDGEWAY, MILL HILL, LONDON, N.W.7

1–2 *November* 1957

The simultaneous determination of both oxygen consumption and contraction response of an isolated muscle preparation employing a system for the continuous circulation of oxygen through the medium.

By A. DARBRE and R. L. SPEIRS. *Departments of Biochemistry and Physiology, King's College, London, W.C.* 2

Oxygen is continuously recirculated through the medium and around the apparatus by means of a moving mercury column acting as a pump. The mercury is raised and lowered either directly by a mercury reservoir attached to a rotating wheel, or indirectly by an air pumping system. The pump is stopped momentarily for the purpose of reading the manometer. A system of

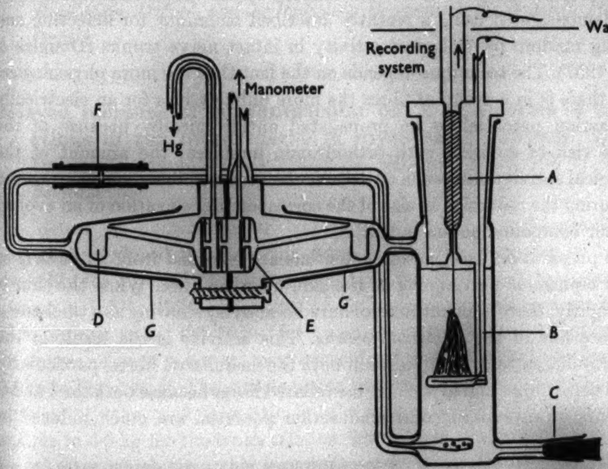

Fig. 1. Diagram of apparatus. *A*, Perspex plug with mercury seal; *B*, clamp electrode holding rat ventricle preparation; *C*, glass side tube with self-sealing rubber plug; *D*, KOH well in Perspex plug; *E*, three non-return latex valves mounted on polythene disks; *F*, rubber sleeve joint; *G*, ground glass sockets. The main chamber and capillary tubes are of glass.

non-return valves allows the gas to pass in one direction only. Carbon dioxide is absorbed as the gas passes over KOH. The clamp electrode and Perspex plug have been described previously (Darbre & Speirs, 1957).

The apparatus is suspended in a thermostatically controlled water-bath (±0·002° C). The side tube on the main chamber allows samples to be taken for analysis and permits the introduction of drugs, salts or other gases into the medium. The volumes of gas and medium are 5 and 10 ml. respectively. This apparatus is simpler in design and construction than that described by Weeks & Chenoweth (1952).

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Demonstration of non-medullated fibres in the saphenous nerve signalling touch.

By W. W. Douglas and J. M. Ritchie. *The Albert Einstein College of Medicine, New York* 61, *N.Y., and the National Institute for Medical Research, Mill Hill, London, N.W.* 7

When the cat's skin is lightly stroked sensory impulses are set up, not only in medullated fibres but also in non-medullated cutaneous afferent fibres. This can be demonstrated using a recently described technique for detecting and measuring random physiological activity in intact nerve trunks (Douglas & Ritchie, 1957). The technique depends on the fact that the more physiological activity there is in a group of fibres the more unlikely it is for an electrically evoked action potential to be propagated antidromically, because of the increased risk of collision with orthodromic impulses; the amount of the physiological activity induced in any particular group of fibres can be measured by measuring the reduction in size of the corresponding elevation of an evoked antidromic compound action potential.

In the preparation used, action potentials are recorded from a fine twig of the cat's saphenous nerve going to the skin near the knee. When the skin is stroked lightly, there is an outburst of nervous activity showing as a thickening of the base line of the electrical records. This activity is the result of the tactile stimulus setting up impulses in both the medullated fibres, particularly the small delta fibres, and in the non-medullated fibres because both the δ and C components of an evoked compound action potential are much reduced in size.

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Defence reactions in the cat elicited by hypothalamic stimulation.

By V. C. Abrahams and S. M. Hilton. *National Institute for Medical Research, Mill Hill, London, N.W.* 7

Effect of impulses in visceral afferent nerves on somatic reflexes.

By M. H. Evans and A. MacPherson. *National Institute for Medical Research, Mill Hill, London, N.W.* 7

Perfusion of the lateral and third ventricles of the cat's brain.

By B. K. Bhattacharya and W. Feldberg. *National Institute for Medical Research, Mill Hill, London, N.W.* 7

A simple calculating device.

By J. L. Malcolm. *National Institute for Medical Research, Mill Hill, London, N.W.* 7

Functional communications between superficial and deep veins in the human forearm.

By D. R. Coles, K. E. Cooper, R. F. Mottram and J. V. Occleshaw. *M.R.C. Group for Research on Body Temperature Regulation, Oxford, and the Radiology Department, the Radcliffe Infirmary, Oxford*

Delayed increase of neuromuscular block in a series of faradizations of the motor nerve in myasthenic patients.

By J. E. Desmedt. *Laboratoire de Pathologie Générale, University of Brussels, Belgium*

The defect of neuromuscular transmission in myasthenia gravis differs from that produced by curarizing drugs in that the block can be increased reversibly for a period of minutes by a brief faradization of the motor nerve (Desmedt, 1957). Related features of the myasthenic junctions are observed when indirect tetani (20–50/sec for 1 sec) are repeated at short intervals. In the course of such a series, neuromuscular function appears to improve as shown in Fig. 1 A, B. The muscle action potentials exhibit less decrement and correlatively the peak isometric tension increases from 2·8 kg for the first tetanus to 3·5 kg for the tenth tetanus. The muscle is now allowed to rest for 1 min, after which time this improvement is no longer obvious (Fig. 1 C). After a further interval of rest of 2 min the response is definitely poorer, tetanic tension being reduced to 2·3 kg (Fig. 1 D). This 'post-tetanic exhaustion' then dissipates progressively in about 15 min.

The sequence of events indicates that two opposing processes with different time courses should be invoked. Post-tetanic exhaustion might result from the depletion and deficient resynthesis of preformed acetylcholine in nerve endings. This phenomenon might be compensated, within limits, by 'post-activation facilitation' which increases the efficiency of the process of acetylcholine ejection in the nerve membrane (see Hutter, 1952; del Castillo & Katz, 1954; Brooks, 1957) and which has a shorter time course. Good and even improved responses should then be observed in spite of a reduced stock of preformed transmitter, so long as the tetani follow each other at short intervals.

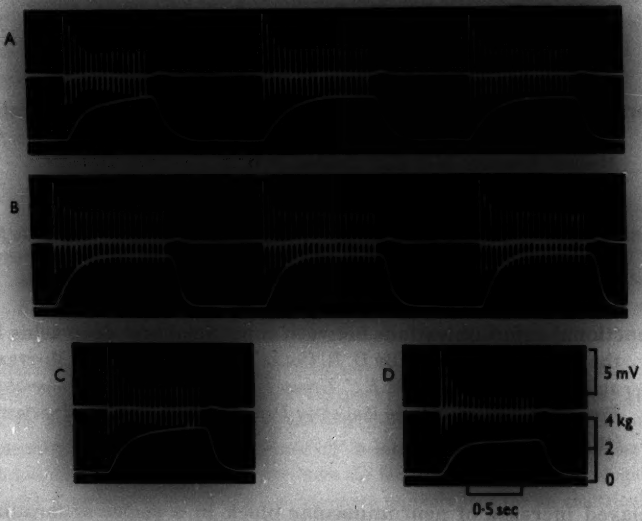

Fig. 1. Patient 21. Moderate generalized myasthenia. Last dose of Prostigmin taken 4 hr before the experiment. Cathode-ray oscillograms of responses of adductor pollicis muscle to supramaximal stimulation of the ulnar nerve at the wrist. The rate is 20/sec. Upper trace, electrical response recorded with belly-tendon surface electrodes. Lower trace, isometric myogram recorded with a strain-gauge myograph. The ulnar nerve is blocked with lignocaine at the elbow. The intra-muscular temperature is 34° C. A, first three responses of a sequence of faradizations separated by intervals of 1 sec; B, 8th, 9th and 10th responses of the same sequence; C, after an interval of rest of 1 min; D, after a further interval of rest of 2 min.

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Experiments on perfusion of the ventricular system of the brain in the cat anaesthetized with chloralose.

By B. K. Bhattacharya and W. Feldberg. *The National Institute for Medical Research, Mill Hill, London, N.W.* 7

The ventricular spaces of the cerebrum were perfused with Locke's solution at 1 ml./10 min from the cannulated left lateral ventricle; the outflow was collected from the cisterna as described recently (Bhattacharya & Feldberg, 1957) or from the aqueduct, so as to confine perfusion to the third and lateral ventricles.

(1) Intravenous tubocurarine, histamine or adrenaline caused great changes in outflow from the cisterna which were at least partly dependent on the blood pressure changes and which usually resulted in an over-all excess outflow over the inflow. Since these changes did not occur on collection from the aqueduct the increased cisternal outflow is unlikely to have been a secretion from the choroid plexus.

(2) Acetylcholine added to the perfusion fluid (with an anticholinesterase) was often only partly recovered from the cisterna, but always 90% or more was recovered from the aqueduct. The partial recovery from the cisterna is explained by the fact that in cats the flow to the subarachnoidal spaces is only via the foramina of Luschka, the foramen of Magendie being absent, allowing an easy mixing of perfusate with the c.s.f. in subarachnoidal spaces outside the cisterna magna.

(3) Adding an anticholinesterase to the perfusion fluid caused the appearance of acetylcholine in the outflowing fluid. The output of acetylcholine was about four times greater with neostigmine than with eserine or DFP. Since collection from the aqueduct reduced the output of acetylcholine only slightly, the structures lining the lateral and third ventricles must be the main source for the released acetylcholine.

(4) On intravenous injection of either eserine or neostigmine in equal amounts, eserine appears in about 40 times larger amounts than neostigmine in the fluid collected from the cisterna when the ventricles are perfused with Locke's solution. This observation agrees with the fact that intravenous eserine elicits central effects but intravenous neostigmine does not. The amounts of eserine appearing in the perfusate decrease to about half when collection is from the aqueduct. It thus seems likely that eserine does not pass into the perfusate through the choroid plexuses but diffuses out of the brain, and that the method of perfusion can give a quantitative estimate of the blood-brain barrier for many drugs.

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Mast cells and anaphylaxis. By I. MOTA. *Department of Pharmacology, University College London*

When chopped lung tissue or a piece of mesentery from a sensitized guinea-pig is incubated with antigen in Tyrode solution at 37° C, a degranulation of mast cells occurs and a reduction in their number. These antigen-induced mast cell alterations show the following characteristics: they are quickly produced and can be detected within 15 sec of contact of the antigen with the tissue; they are produced by low concentrations of antigen such as 10^{-7} ovalbumin; they increase with time and with concentration of the antigen; they are very temperature-dependent, being maximal at 40° C and completely inhibited at 15° C; they are completely prevented when the sensitized tissue has been heated to 45° C before addition of antigen; they are completely inhibited by lack of calcium; they are partially inhibited by iodoacetate and phenol.

When these mast cell alterations are compared with those produced by octylamine, a histamine releaser with high activity, several differences are apparent. Morphologically the alterations induced by octylamine are clearly different from those induced by antigen. They are characterized by fusion and clumping of the granules so that the mast cell looks like a blot; there is no degranulation such as occurs with antigen. The mast cell alterations are not prevented when the tissue has been previously heated to 45° C, and they are not inhibited by lack of calcium, iodoacetate, or phenol.

There is a close parallelism between the effects of these various agents on mast cells and on histamine release. Iodoacetate, phenol, pre-treatment of the tissue at a temperature of 45°, and lack of calcium inhibit histamine release (Mongar & Schild, 1957 *a–c*) as well as mast cell alterations by the antigen, but they do not inhibit histamine release and mast cell alterations by octylamine. Since mast cell alterations are produced by the action of the antigen both *in vivo* (Mota & Vugman, 1956; Mota, 1957) and *in vitro*, it seems probable that the mast cells contain antibody and play an important role in anaphylaxis.

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Patterns of discharge of single units of the lateral geniculate body of the cat in response to binocular stimulation. By S. D. ERULKAR and MARIANNE FILLENZ. *University Laboratory of Physiology, Oxford*

This study is concerned with the reactions of single units of the lateral geniculate body of the cat to stimulation of the eyes with light flashes. Two types of binocular effect have been observed. (1) A particular unit may be activated by stimulation presented to either eye. The pattern of response may, however, vary according to which eye is stimulated. We have recorded from seven units which behave in this manner. (2) Other units, of which thirteen have been studied, discharged in response to stimulating one eye, but not to separate stimulation of the other. However, the pattern of discharge of such units is modified if the other eye is stimulated during the response. The modification of the discharge consists of changes in the latency, the frequency of firing, the durations of pauses in the response, and the duration of the response itself. The time relationships of the two stimuli are important.

Undoubtedly there are units whose activity is influenced exclusively by stimulation of one eye alone, but we do not possess accurate data on the proportion of these units. We conclude, however, that some convergence of fibres from both sides on to single units takes place at the lateral geniculate body of the cat; furthermore, that the patterns of impulses being relayed to the cortex in response to stimulation of one eye can be modified by stimulation presented to the other eye. These conclusions are in agreement with those of Bishop & Davis (1953), who recorded wave responses from the optic radiation of the cat.

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Effects of catechol amines present in peripheral tissues. By J. H. Burn and M. J. Rand. *Department of Pharmacology, University of Oxford*

Noradrenaline and adrenaline are normally present in the heart. When rabbits are treated with reserpine, these substances are liberated from the heart, only traces remaining (Bertler, Carlsson & Rosengren, 1956). When dogs are treated with reserpine similar observations have been made: the effects of these substances have been recorded during their liberation (Paasonen & Krayer, 1957; Innes & Krayer, 1957).

Kottegoda (1953) observed that nicotine caused an increase in the rate and amplitude of the isolated atria of the rabbit when the nicotine was applied in the presence of atropine. We have now observed that when the atria are taken from rabbits treated with reserpine nicotine has no stimulant action. The stimulant action of nicotine may therefore be attributed to the release of catechol amines.

Kottegoda also observed that nicotine (and ACh in the presence of atropine) had a constrictor action on the vessels of the perfused rabbit ear. This constrictor action appeared to be due to the release of catechol amines, since when Priscol was present in the perfusing fluid, the constrictor action was converted to a dilator action, just as was the constrictor action of noradrenaline and adrenaline. Ginzel & Kottegoda (1953) showed that the constrictor action of nicotine was unaffected by degeneration of all sympathetic fibres to the ear after removal of the stellate and superior cervical ganglia.

We have now observed that in rabbits treated with reserpine nicotine has no longer any constrictor effect on the perfused vessels of the ear, nor has ACh in the presence of atropine. This evidence confirms the suggestion of Kottegoda that nicotine causes constriction by releasing catechol amines.

Some human subjects suffer from thromboangiitis obliterans with loss of the tips of the fingers. Their attacks are brought on by smoking (McDevitt & Wright, 1955). Nordenstam & Adams-Ray (1957) have described the presence of chromaffin tissue in human skin. In view of the above findings this disease may be explained by a hypersensitivity to the action of nicotine in releasing catechol amines located near the blood vessels of the skin.

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Helical configuration of smooth myofibrils.

By Jean Hanson and J. Lowy. *Medical Research Council Biophysics Research Unit and Wheatstone Physics Laboratory, King's College, London, W.C.* 2

The appearance of 'double-oblique striation' in the muscle fibres of many different invertebrate animals has been known for a long time, but its meaning is still obscure. Ballowitz (1892), Marceau (1905, 1909) and others considered that it is due to helically wound smooth myofibrils; however, more recently, Plenk (1924, 1933) and Schmidt (1938) concluded that it is really a distorted cross-striation. We have examined fibres from a variety of these muscles in the phase-contrast microscope and, after glycerol extraction, observed them shortening during treatment with adenosine triphosphate (ATP). Earthworm muscles (Hanson, 1957) and squid muscles (Hanson & Lowy, 1957) were also studied by electron microscopy. It has been found that in some of these muscles the appearance of 'double-oblique striation' is due to helically wound smooth myofibrils; but in others (e.g. certain lamellibranch adductors) the 'striation' only appears when the muscle is made to shorten below minimal body length, and is then seen as a rather variable pattern of oblique lines superimposed on typical smooth-muscle structure. We consider that all the muscles in which a 'double-oblique striation' has been described are in fact smooth muscles, and that the term should be abandoned.

One of the most interesting things about these muscles is that in some of them the myofibrils are helically arranged. In squid mantle muscles, for example, each fibre has a core of sarcoplasm around which is wound a single set of ribbon-shaped myofibrils which are not differentiated in any way along their lengths. The ribbons lie radially, with one edge at the surface of the fibre and the other edge next to the sarcoplasmic core. The helix is left-handed, and in unshortened muscle the angle it makes to the fibre axis is about 5°. The angle is greater in contracted muscle, and in isolated glycerol-extracted fibres it can increase to about 60° during ATP-induced contraction.

A helical arrangement of fibrils is found not only in the fast mantle muscles but also in the gut muscles of the squid. Therefore it cannot be regarded as a specialization of smooth muscle for rapid contraction.

H. E. Huxley (personal communication) has pointed out that a muscle fibre with helically arranged contractile elements could develop tension and shorten if the core around which the helix is wound remains at constant volume and if the fibre cannot twist. This is a useful working hypothesis because it can be assumed that the volume of the fibre remains more or less constant during contraction, and histological examination suggests that the fibres in the muscle are effectively anchored by connective tissue.

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Biosynthesis of 5-hydroxytryptamine. By M. Sandler and G. B. West.

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There is clear evidence that 5-hydroxytryptamine (5-HT) is formed in the body by decarboxylation of 5-hydroxytryptophan (5-HTP), but the distribution of the decarboxylase differs from that of the 5-HT. In the rat, for example, the kidney possesses high enzyme activity and a low 5-HT content, whereas the spleen has little or no enzyme activity and a relatively high 5-HT content (Gaddum & Giarman, 1956). The gut contains both high enzyme activity and a relatively high concentration of 5-HT.

We have studied in perfusion experiments the conversion by rat tissues of 5-HTP to 5-HT. Preparations of hind quarters, gastro-intestinal tract or kidney were perfused with oxygenated Locke solution. Injections of 1 mg DL-5-HTP (with added pyridoxal phosphate) were made into the arterial cannulae and the venous effluent was collected over 2 hr and assayed for 5-HT on the atropinized rat's uterus. Decarboxylation in the hind quarters was less than 0·1%, but in the gut and kidney it was about 1%. Iproniazid (an inhibitor of monoamine oxidase) failed to increase the total amount of 5-HT in the perfusate, although it brought forward the peak of 5-HT release. In rats whose tissue histamine had previously been depleted by treatment with polymyxin B (Parratt & West, 1957), there was also no increased output of 5-HT. Thus reduction of tissue histamine and disruption of mast cells do not alter 5-HTP metabolism, as measured by this method. On the other hand, the yield of 5-HT was almost doubled in all three preparations following reserpine pre-treatment. Reserpine is known to inhibit the binding of 5-HT by the tissues (Brodie, Pletscher & Shore, 1955). Work is now in progress to study metabolites in the tissues and perfusate using ^{14}C-labelled 5-HTP.

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Estimation of 5-hydroxytryptamine in blood and serum. By HELEN E. C. CARGILL-THOMPSON, D. C. HARDWICK and JEANNE M. WISEMAN. *A.R.C. Institute of Animal Physiology, Babraham, Cambridge*

Several workers (e.g. Erspamer, 1954; Bigelow, 1954) have estimated the 5-hydroxytryptamine (5-HT) content of serum. Hardisty & Stacey (1955) calculated for human blood, and Humphrey & Jaques (1954) for other species, that serum did not contain all the platelet 5-HT of the blood. Attempts to measure the whole blood 5-HT by acetone extraction have yielded low values (Toh, 1954; Hardisty & Stacey, 1955).

We have used the extraction method of Udenfriend, Weissbach & Clark (1955) for blood with the following slight modifications. 1·5 ml. heparinized blood was mixed with 1·5 ml. water, added to the buffer-butanol mixture and shaken by hand for 2 min. No pH adjustment was made to the haemolysed blood, and in our experience the pH of the aqueous layer should be 8·7–9·2 for maximum 5-HT extraction. The butanol phase was not washed, but extracted at once with heptane 0·1 N-HCl mixture. The aqueous layer was washed twice with 5 ml. ethyl ether and traces of ether removed by blowing air through the solution for 2–3 min. The solution was then neutralized and made up to 5 ml.

with the necessary salts to give concentrations as for de Jalon's solution (Gaddum, Peart & Vogt, 1949). The solution was assayed on the superfused oestrous rat's uterus. BOL-148 or LSD-25 were used to inhibit 5-HT, and blood and serum extracts behaved as if 5-HT were the only agonist present. Average recovery of 0·4–0·8 μg 5-HT was 73%, and all the values have been corrected correspondingly.

Adrenaline did not interfere when 2 μg was added to the initial solution containing 0·8 μg 5-HT.

Using this method, we found that good recoveries (71–139%, average 92%) of 5-HT added to whole blood could be obtained in some species (cat, dog, goat, ox, rat and possibly horse). Where 5-HT had been added to blood and this had been allowed to clot, recovery from the serum was poor (21–54%, average 35%). In other species (guinea-pig, human, pig, rabbit and sheep) when 5-HT was added to whole blood, recoveries were low, both from whole blood and from serum (whole blood: 39–96%, average 56%; serum: 24–58%, average 33%). In all species, the recovery of 5-HT added to serum was good (75–140%, average 96%).

The observed serum values were lower than expected from the whole blood and haematocrit values; experiments indicated that adsorption on to red cells occurred in human and goat blood.

We acknowledge a gift of BOL-148 from Dr Holgate of Sandoz Products Ltd; the LSD-25 used was a gift of Dr W. Feldberg to Dr J. L. Linzell.

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The effect of adrenaline and acetylcholine on the activity of single neurones in the reticular formation of the brain. By P. B. Bradley* and A. Mollica. *Institute of Physiology, Pisa, Italy*

The effects of adrenaline and acetylcholine on unit activity in the reticular formation have been studied in decerebrate cat preparations. Recordings were made both from the mesencephalon and from the medulla using a floating micro-electrode technique, and blood pressure was recorded from the carotid or femoral artery. The unit activity and blood-pressure level were displayed simultaneously on a two-channel cathode-ray oscillograph and photographic records of these phenomena made on moving film. In the mesencephalon only units showing convergence responses were selected, but in the medulla the activity of some non-convergent units was examined as well. In certain experiments the effect of the drugs was also tested on a number of units in specific sensory projection systems.

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When adrenaline was injected intravenously (5–25 μg) or into the common carotid artery (1–5 μg) the activity of many units was unaffected, but others showed a change in their discharge rate which was either an increase in frequency, or a decrease. With intravenous injections the changes in unit activity usually accompanied the rise in blood pressure, whereas with intracarotid injections no blood pressure changes occurred, although similar changes in the activity of many reticular units were observed.

Acetylcholine (0·2–1·0 μg) was injected only by the intracarotid route and all reticular units tested with this drug showed a response. This response was twofold, consisting of a primary increase or decrease in frequency which started immediately, or within a few seconds of the injection and without change in blood pressure; and a secondary effect with a much longer latency (20–24 sec). The latter was also either an increase or decrease in frequency but it was invariably accompanied by a slight fall in blood pressure. The secondary response was more constant in its appearance and was observed with every unit tested, whereas the primary response was not always seen.

The effects of both drugs on unit activity were still present after bilateral denervation of the carotid sinus and also after the injection of a paralysing dose of curare. No changes in the activity of units in specific sensory pathways were observed with these drugs, even when larger doses were used.

The effects of intracarotid injections of adrenaline and also the short-latency effects observed with acetylcholine are probably produced by direct central actions of these drugs. The results suggest that there may be elements present in the reticular formation which are sensitive to either or both of these drugs, whereas other elements appear to be insensitive. The variety of responses which can be observed indicates the complexity of the neuropharmacological mechanisms which exist in this part of the brain.

Urinary excretion of histamine in pregnant rats.

By G. Kahlson, Elsa Rosengren and H. Westling. *Institute of Physiology, University of Lund, Lund, Sweden*

It has been shown that rats, raised and fed on a synthetic, histamine-free diet have the same pattern of tissue histamine and urinary excretion of this amine as germ-free reared rats similarly fed (Gustafsson, Kahlson and Rosengren, 1957). These rats, which are not germ free, appear useful for the study of physiological conditions where histamine might be involved. In such rats an increase in the urinary excretion of histamine is not likely to be masked by a high basal excretion of histamine mainly of exogenous origin (histamine in the food, histamine formed by bacterial decarboxylation of histidine in the gut).

The daily urinary excretion of histamine was examined before, during and after pregnancy in a series of normally kept rats fed on the synthetic, histamine-free diet. In four rats the mean histamine excretion before pregnancy was 18, 19, 24 and 43 μg (base) in 24 hr. During the first 14 days of pregnancy no conspicuous change was noted. At the 15th day, in each rat, the urinary output started to increase. This increase progressed to reach a peak 1 or 2 days before parturition. The mean values for daily histamine excretion during the last 8–10 days of pregnancy were 131 (6), 290 (9), 271 (8) and 74 (1) μg/24 hr. The figures in parentheses denote the number of young. It appears that the output of histamine was related to the number of foetuses. The highest values observed for the daily output were 228 (6), 835 (9), 536 (8) and 123 (1). At 1 or 2 days before parturition the urinary histamine fell steeply to the non-pregnant level.

In three rats the effect of the histaminase inhibitor aminoguanidine was studied during a second pregnancy. The inhibitor was given subcutaneously in daily doses of 20 mg/kg. This treatment resulted in an increase of urinary histamine before and during pregnancy. In three rats given the inhibitor the mean daily excretion of histamine before pregnancy was 76, 86 and 104 μg, during the first 14 days of pregnancy no conspicuous further increase occurred and during the last 8–9 days the mean daily output was 503 (4), 381 (4) and 760 (6). The highest single value noted was 1620 (6). Again, the figures in parentheses denote the number of young. All the figures given refer to free histamine. There was no evidence of conjugated histamine in the urine in these experiments.

The cause of the increased output of histamine is so far unknown and subject to further studies.

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Gonadotrophin incompatibility within species and strains.

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Hormone incompatibility *between* species constitutes an important problem in endocrine therapy, and, in particular, incompatibility of gonadotrophins from various animal sources has been the subject of detailed studies (Zondek & Sulman, 1942). The heterologous gonadotrophin leads to the suppression of the pituitary by some unexplained mechanism.

Although tissue antigens and blood-group differences are the rule *within* species, incompatibility of homologous hormone extracts has not so far been encountered (Leathem, 1949). While investigating the effect of cryptorchism on intact male and female parabionts, however, results which suggest such a situation have been obtained.

Parabiosis between intact and intact or intact and cryptorchid male litter-mate albino mice (ordinary stock) often resulted in varying degrees of testicular atrophy, spermatogenic arrest, interstitial-cell hypoplasia and shrinkage of the seminal vesicles at 2–6 weeks. The mean weight of the testis in 47 intact parabionts was 75 ± 3·6 mg, while the mean for 47 controls of the same weight was 96 ± 2·0 mg ($t = 5{\cdot}08$, $P \ll 0{\cdot}001$; $F = 3{\cdot}40$, $P < 0{\cdot}001$).

In the intact females, of 67 ovariectomized female with intact female, cryptorchid male with intact female, and intact male with intact female pairs, partial or complete suppression of oestrus occurred. This bore no relation to parabiosis intoxication and ill mice frequently exhibited strong oestrus. Oestrus suppression was associated with macroscopic and histological ovarian atrophy and was more pronounced in non-litter-mate parabionts. Seven pairs in which the intact members showed continued anoestrus were separated at 6 weeks and all resumed regular cycles within 5–20 days. Cortisone acetate (0·25 mg) given daily for a fortnight had no effect, but normal response occurred after a single dose of 0·1 μg oestradiol and also after 0·25 i.u. of human chorionic gonadotrophin given daily.

The pure strain C_3H and hybrids of pure strains (C 57 BL + Balb/C)—12 pairs—showed no evidence of gonadotrophin incompatibility either in the males or females. All intact females of 10 male with female C 57 BL parabionts exhibited partial or complete oestrus suppression, suggesting sex-linked gonadotrophin within this particular strain.

The importance of these results in the testing of hormone extracts (established or hypothetical) in impure strains is evident.

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The innervation of mammalian neuromuscular spindles. By

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The generally accepted picture of mammalian spindles is that of Barker (1948). Usually it is assumed, in view of the work of Hunt & Kuffler (1951), that the intrafusal fibres are tonic, probably with multiple innervation by small nerve fibres (γ-efferents), and without a propagated action potential. The present study of spindles, using gold chloride, shows that the structure is more complex than this.

The spindles of the cat's tenuissimus muscle were described previously (Boyd, 1956); they contain intrafusal muscle fibres of two distinct sizes, small-diameter fibres each about half the total length of the spindle, and large-diameter fibres which stretch from end to end. This appears also to hold for other mammalian spindles. No intrafusal fibres have been seen to divide. In the equatorial region of the spindle the large fibres have a nuclear bag, while each of the small fibres has a chain of nuclei down the centre.

Each spindle has a primary (annulo-spiral) sensory ending in the nuclear region, with spirals round each large and small muscle fibre, supplied by an axon of large diameter. There may be either two secondary (flower-spray) endings, one towards each pole at a variable distance from the primary ending, or only one secondary ending, or occasionally none at all. This ending has sprays on both large and small muscle fibres, supplied by an axon of medium diameter.

The motor supply of the large intrafusal fibres consists of one or two (occasionally more) discrete end-plates, similar to extrafusal end-plates, on each polar half of each fibre. The axons are of medium diameter and often accompany bundles of motor axons to extrafusal fibres, but no one axon has been seen to supply both extrafusal and intrafusal muscle fibres. No end-plates of this type have been seen on the small intrafusal muscle fibres.

Another type of ending occurs exclusively on the small intrafusal fibres, polar to the secondary sensory ending. It has a network of densely staining particles connected by fine nerve fibrils, like the flower-spray ending, but the particles are in chains rather than in sprays. In larger spindles these chains extend along individual small muscle fibres through the entire sensory region. Their axons are of similar diameter to the motor axons to the large fibres. Nerve degeneration experiments are now being undertaken to determine whether these endings are motor or sensory. If motor, they may be analogous to the endings on the slow (tonic) fibres of lower animals.

It is suggested that the large intrafusal fibres may form a 'twitch' mechanism, and the small fibres a 'tonic' mechanism. Experiments with micro-electrodes on isolated spindles are in progress to test this hypothesis.

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Active muscle vasodilatation and its relation to the 'Flight and Fight reactions' in the conscious animal.

By V. C. Abrahams* and S. M. Hilton. *The National Institute for Medical Research, Mill Hill, London, N.W. 7*

Lindgren, Rosen, Strandberg & Uvnäs (1956) have shown that electrical stimulation of certain regions n the hypothalamus, mesencephalon and medulla produce vasodilatation in skeletal muscle, thus providing evidence for a vasodilator pathway in the brain stem. This vasodilatation is due to activation of cholinergic vasodilator fibres (Eliasson, Folkow, Lindgren & Uvnäs, 1951). In the present experiments on anaesthetized cats the hypothalamic vasodilator pathway has been localized more precisely, using unipolar stimulating electrodes of 8–25 μ tip diameter.

Muscle vasodilatation was readily elicited on stimulation of the anatomically undifferentiated grey matter extending from the pre-optic area anteriorly to the mesencephalic central grey matter posteriorly. The excitable zone consisted, on either side of the mid line, of a horizontally flattened strip between the anterior commissure and the supraoptic nucleus in the pre-optic area. It continued posteriorly into the hypothalamus just ventral and medial to the fornix, in some experiments lateral to it also. The zone then continued further as a vertically flattened strip just dorso lateral to the mammillary body and ascended into the central grey matter.

Eliasson *et al.* (1951) noted that the muscle vasodilatation was often but not always accompanied by vasoconstriction in the skin and intestine, tachycardia, rise in blood pressure, pupillary dilatation and retraction of the nictitating membranes; they suggested that the muscle vasodilatation represented a reaction in preparation for muscular exercise. We observed these accompanying effects regularly, and, in addition, saw pilo-erection and widening of the palpebral fissure. In general the more pronounced the muscle vasodilatation, the more prominent were the associated effects.

It is known that stimulation of a localized area in the hypothalamus elicits the 'defence reaction' in the conscious cat (Hess & Brügger, 1943). This localized area appeared to be identical with the hypothalamic zone from which we had obtained our responses in the anaesthetized animal. Accordingly, we stimulated in the conscious cat, with an electrode chronically implanted in the hypothalamic zone from which muscle vasodilatation was elicited, and the typical picture of the 'defence reaction' was obtained. Immediately, the pupils dilated and the animal was alerted; after a few seconds it hissed and snarled, and started to run round the cage. There was massive pilo-erection. If disturbed by a sudden noise, the cat would start to attack in the direction of the sound. Stimulation via the chronically implanted electrode under chloralose anaesthesia produced the cardiovascular responses, including the characteristic muscle vasodilatation, and the other sympathetic reactions. It therefore appears that all these responses obtained under anaesthesia, and particularly the pattern of vasodilatation and constriction, are part of the general 'defence reaction', being preparatory to the muscular exertion of flight or fight.

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The interaction of evoked cortical responses.

By J. L. Malcolm and I. Darian Smith.* *The National Institute for Medical Research, Mill Hill, London, N.W.* 7

The extent to which cortical neurones are excited by the arrival of an afferent volley depends upon the nature and strength of the peripheral stimulus, and the type of anaesthetic used (Adrian, 1941; Marshall, Woolsey & Bard, 1941).

In cats under chloralose anaesthesia, stimulation (by an electrically controlled crystal movement, cf. Gray & Malcolm, 1949) of the touch receptors of discrete areas of skin of the contralateral fore and hind paws, regularly excites cortical areas similar to those excited in cats under barbiturate anaesthesia, but the area involved and the amplitude of the evoked responses is dependent upon the strength of the peripheral stimulus.

In somato-sensory area II the representation of the contralateral fore and hind paws overlaps extensively, while in somato-sensory area I the overlap is slight or absent.

The extent to which this overlap in cortical representation is brought about by common pathways was investigated by observing the interaction of two successive afferent volleys. When the two volleys are initiated at the same peripheral point, the occlusion of the cortical response to the second volley is most complete, is maintained over the longest time interval and is similar in somato-sensory areas I and II. When the volleys are initiated at points as far apart as the fore and hind paws the pattern of occlusion is different. In somato-sensory area II, where the overlap of the area of representation of these two points is most marked, considerable occlusion can still be demonstrated. In somato-sensory area I, where the overlap is slight, occlusion is minimal or absent.

The site of the common pathway involved in the interactions of long duration is probably not in the cortex. This is suggested by the interaction of the response evoked by touch with the response evoked by stimulation of the contralateral cortex and transmitted by the fibres of the corpus callosum.

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Elements common to these two pathways can reside only in the cortex in the region of the recording point. Occlusion between volleys in these two pathways occurs only during very short time intervals, demonstrating common cortical elements which cannot be involved in the longer interaction between volleys from two peripheral points.

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Effect of electrical stimulation on the concentration of phosphorylated intermediary metabolites in mammalian non-myelinated fibres.

By P. Greengard and R. W. Straub. *The National Institute for Medical Research, Mill Hill, London, N.W. 7*

Corresponding pieces of the two cervical vagi of rabbits were cleaned under the dissecting microscope, cut to equal length (4–5 cm), mounted on platinum electrodes and shown to conduct impulses by monitoring the compound action potential. One vagus was then stimulated at 6, 15 or 50/sec with stimuli supramaximal for C fibres and, after 15 sec, while still being stimulated, was plunged into a tube containing 1·5 ml. of 0·1 M triethanolamine buffer at 100° C and pH 8·1. After 40 sec the tube was cooled rapidly to 0° C and the contents homogenized by means of a glass pestle ground to fit the lower part of the tube. Next, 1·5 ml. of alcohol-free chloroform was added, the tube stoppered, shaken vigorously for 3 min and centrifuged at 3000 *g* for 10 min. The clear supernatant was then removed and analysed for intermediary metabolites by fluorimetric enzyme methods (Greengard, 1956). Concentrations of intermediary metabolites were calculated with respect to protein content of the nerves. The precipitated protein was estimated, after removal of the chloroform, by the method of Lowry, Rosebrough, Farr & Randall (1951). In each experiment the second vagus served as unstimulated control.

It was found that the non-stimulated vagi contained, per mg protein, approximately $4{\cdot}0 \times 10^{-9}$ mole of creatine phosphate (CrP), $5{\cdot}0 \times 10^{-9}$ mole of adenosine triphosphate (ATP), $0{\cdot}6 \times 10^{-9}$ mole of adenosine diphosphate (ADP) and $0{\cdot}1 \times 10^{-9}$ mole of adenosine monophosphate (AMP). In three experiments in which unstimulated pairs of nerves were compared the levels of CrP, ATP, ADP and AMP agreed within 5 %. When one vagus was stimulated at 6/sec (three experiments) or at 15/sec (five experiments) the levels of CrP, ATP, ADP and AMP also agreed, within 5 %, with the unstimulated controls. However, in nine experiments in which one vagus was stimulated at 50/sec, there was a decrease of 15–30 % in the content of CrP and of ATP. In two of these experiments the loss of ATP was fully accounted for by an increase in the levels of ADP and AMP. This increase was absent in the other experiments, suggesting that the newly formed ADP and AMP had further broken down.

The amounts of glucose-6-phosphate and phosphoenolpyruvate were less than 5×10^{-11} mole/mg protein for both resting and stimulated nerves.

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After-potentials following a single impulse in mammalian non-myelinated fibres. By P. GREENGARD and R. W. STRAUB. *The National Institute for Medical Research, Mill Hill, London, N.W.* 7

The after-potentials following a monophasic spike were studied on desheathed bundles of non-myelinated fibres obtained from one of the subdivisions of the rabbit's cervical sympathetic trunk, which were mounted in the apparatus described elsewhere (Stämpfli, 1954; Ritchie & Straub, 1957). With the fibres in Locke's solution the spike was followed by a negative after-potential, which declined approximately exponentially with a time constant of 150–400 msec at 37° C. Changing the potassium concentration in the bathing solution did not affect the time constant. Lowering the temperature by 10° C over the range from 39° to 8° C increased the time constant about two fold in fresh preparations and 1·4-fold in older ones. During a train of impulses there was a progressive build-up of the negative after-potentials. These results are similar to those described by Frankenhäuser & Hodgkin (1956) on giant nerve fibres. This similarity suggests that the negative after-potential in C fibres can be explained, as shown for the giant nerve fibres, by accumulation of the potassium ions released during the spike in a space around the fibre membrane.

In each experiment the increase in the potassium concentration near the excitable membrane was estimated from the curve which relates the resting potential to the potassium concentration in the bathing fluid. The increase in potassium concentration immediately after the spike amounted to 3·4 ± 0·6 mM (S.D. 4 experiments) at 22–26° C. The space between nerve membrane and Schwann cell described by Gasser (1955), measures about 140 Å (J. D. Robertson, personal communication). Assuming that the potassium accumulates only in this space, the potassium outflow per impulse calculated by multiplying the rise in potassium concentration by the thickness of the space is $4\text{–}5 \times 10^{-12}$ mole cm^{-2}.

Following the negative after-potential there was a small positive after-potential, which reached its peak about 800 msec after the spike. This after-potential was abolished when the fibres were stimulated in a solution in which the sodium ions had been replaced by lithium ions. This finding suggests that the positive after-potential following a single impulse is caused by an increased activity of the sodium-potassium pump in a manner analogous to that described for the post-tetanic hyperpolarization by Ritchie & Straub (1957).

In potassium-free solution and at temperatures below 27° C there was, in addition, a brief period of hyperpolarization preceding the negative after-potential. This initial hyperpolarization is probably identical with the 'positive phase' observed on isolated giant axons (cf. Frankenhäuser & Hodgkin, 1956).

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Anaesthesia and sleep-like conditions produced by injections into the cerebral ventricles of the cat.

By W. FELDBERG. *The National Institute for Medical Research, Mill Hill, London, N.W. 7*

Adrenaline, noradrenaline and $CaCl_2$ produce anaesthesia-like conditions on injection into the cisterna magna or the lateral ventricle (Bass, 1914; Marinesco, Sager & Kreindler, 1929; Leimdorfer, 1950; Feldberg & Sherwood, 1954, 1957; Reitter, 1957; Haley & McCormick, 1957). Since these substances are neither anaesthetics nor hypnotics, the problem arose of how substances belonging to these groups would act when injected intraventricularly into the unrestrained conscious cat. The results were as follows:

$MgCl_2$ (2–5 mg) produced an anaesthesia-like condition lasting about 1 hr and associated with hyperexcitability. Ear tickling led to vigorous ear flicker, sometimes accompanied by repeated head shaking, and once by a convulsive spasm.

Urethane (20–30 mg) caused a condition resembling sleep. Sometimes the cats curled up with eyes closed; they were easily startled by noise and fully roused by firm stroking. Before falling asleep and before coming round, there was severe ataxia, restlessness and hyperphagia or bulimia. Unable to stand or sit, with the head shaking or rocking, they would voraciously devour meat.

Chloralose produced anaesthesia with pronounced slowing of respiration, protrusion of nictitating membrane and sometimes abolition of blink and corneal reflexes. In the first experiment, with 4 mg, deep anaesthesia ensued for 5 hr; respiration stopped after 45 min, necessitating rhythmic thoracic massage for 2 hr. When coming round an 'overactive arousal reaction' developed, followed by severe ataxia whilst the cat devoured a full pot of meat. With 1–2 mg, anaesthesia lasted 1½–2 hr; with 0·2 mg a condition more like sleep was produced; anaesthesia and sleep were preceded and followed by periods of restlessness, ataxia and hyperphagia during which the cats were very affectionate. The results show that with intraventricular injections of chloralose it is possible to study physiological and pharmacological reactions in anaesthetized animals without large amounts of the anaesthetic circulating in the blood.

Barbiturates seemed at first unsuitable for injection, being soluble in strong alkali only. However, 4–5 mg pentobarbitone sodium was soluble at about pH 8 in 1 ml., and the injection of 0·3 ml. of this solution produced anaesthesia in some cats, a sleep-like condition in others, and in yet others, neither anaesthesia nor sleep developed, but in all there was initially severe ataxia, restlessness and hyperphagia; this happened again, in those showing anaesthesia or sleep, when coming round.

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A transient, periodic secretion from the parotid gland after post-ganglionic, parasympathetic denervation.

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In cats under chloralose anaesthesia there is ordinarily no flow of saliva from the parotid glands. After section of the auriculo-temporal nerves, however, we have observed a secretory activity of characteristic type. During a period of about 3 days, starting about 1 day after the section of the nerves, outbursts of secretory activity suddenly occur, alternating with periods of inactivity. Several drops of saliva may fall from the cannula inserted in the parotid duct, in the course of some minutes; then a period of rest, lasting for 1–2 hr, ensues. Even during the period of activity the flow is irregular; a drop increasing slowly in size may suddenly be seen to grow quickly for a short time.

The phenomenon is found after post-, but not after preganglionic, parasympathetic denervation. After bilateral section of the auriculo-temporal nerves the secretory activity of one gland does not coincide in time with that of the other one; the intermittent activity thus seems to be connected with local events in the gland. It is abolished by atropine, injected intravenously, and, likewise, by cocaine, injected into the parotid duct in a dose which does not diminish the secretory effect of acetylcholine given intravenously.

Eserine, injected through the salivary duct of the normally innervated gland causes a flow of saliva at a regular, slowly decreasing rate. Five days, or more, after section of the auriculo-temporal nerves the secretory effect of eserine is very small. If the drug is given on the second to fourth day after the operation, however, it evokes a lively secretion; the flow is much bigger than that seen at the same stage with no eserine given, but resembles it in being periodic.

It seems reasonable to assume that the transient, periodic secretion following section of the auriculo-temporal nerves is due to acetylcholine, released in outbursts from the endings of the degenerating post-ganglionic parasympathetic nerve fibres.

Release of sympathetic transmitter from the intestine of the cat.

By G. L. Brown, B. N. Davies* and J. S. Gillespie†. *Department of Physiology, University College London*

Brown & Gillespie (1957) have shown that the amount of noradrenaline appearing in the splenic venous blood is greatly increased by the administration of a sympathetic blocking agent at frequencies of nerve stimulation less than 30/sec. The output of noradrenaline was not affected by the administration of an antiamine-oxidase. The spleen was chosen because noradrenaline could easily be detected in the venous blood on stimulating the splenic nerves. Several other organs were abandoned because no transmitter appeared in the venous blood. We have now re-examined the intestine of the cat to see if blocking the receptors for noradrenaline would affect the output.

The sympathetic post-ganglionic nerves to the colon or to the small intestine were stimulated with known numbers of supra-maximal shocks. The venous blood from the intestine was collected during and after nerve stimulation. This venous blood was centrifuged and the noradrenaline content of the separated plasma was assayed on the blood pressure of the pithed rat.

We have confirmed our previous finding that in both the colon and the small intestine nerve stimulation in the absence of a blocking agent does not produce significant amounts of noradrenaline in the venous blood. If, however, dibenzyline (*N*-phenoxy*iso*propyl-*N*-benzyl-β-chloroethylamine) 10 mg/kg is given and nerve stimulation repeated, the tissue response is abolished and noradrenaline in easily measurable amounts appears in the venous blood. If after blocking the receptors in this way, Marsilid (Iproniazid) is given to inhibit monoamine oxidase, and nerve stimulation is repeated, there is no increase in the output of noradrenaline. It seems then that in the intestine, as in the spleen, the liberated transmitter is inactivated after its combination with the receptors.

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By collecting separate successive blood samples during and after nerve stimulation we have been able to follow the removal of the transmitter from the tissues. This removal is relatively slow, which may explain the greater effectiveness of inactivation in the intestine as compared with the spleen.

We have also investigated the effects of repeated, prolonged stimulation of the nerves. They were stimulated at 50/sec for 100 sec before we began to collect a sample, and stimulation was continued for the duration of collection, usually a further 20 sec. These 2 min periods of stimulation were repeated at 10 min intervals, and the output at the end of each was found to decline progressively to reach a base line of about 10–20% of that in the first period. If the interval between periods of nerve stimulation was now increased from 10 min to 3 hr, there was almost complete recovery of the output of transmitter. The likeliest explanation of this reversible decline in the output is that the stores of transmitter at the nerve endings are depleted.

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Effects of drugs and anaesthetics on reanimation of ice-cold mice.

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Reduction of deep body temperature to about 20° C has been shown to protect non-narcotized new-born and adult animals from lethal exposures to asphyxia (Miller & Miller, 1954) and is even more effective in narcotized animals. Below 10° C metabolic requirements approach the vanishing point, and it would be advantageous to cool animals to this degree in any conditions in which there is tissue hypoxia.

Andjus & Smith (1954) demonstrated that high percentages of rats can be resuscitated from temperatures near zero. A feature of their technique was that the animals were cooled slowly in sealed jars to body temperatures of about 15° C. They were thus subjected to a combination of hypoxia and accumulation of CO_2. Goldzveig & Smith (1956) obtained equally high percentages of revivals with a simpler procedure for reanimation after the same method of cooling.

Recently, rats and larger mammals have been revived from body temperatures below 5° C (Niazi & Lewis, 1954, 1957) without exposing them to the combination of decreasing oxygen and increasing carbon dioxide tensions. This makes it desirable to determine whether any aspect of the closed vessel technique is essential for subsequent recovery and whether similar results can be obtained with small animals cooled by other methods.

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Experiments to date show that it is possible to resuscitate mice which have been cooled rapidly to body temperatures between 0 and 1° C after treatment with general anaesthetics (ether, chloroform, nitrous oxide and oxygen), barbiturates (Nembutal, pentothal), a tranquillizing drug (chlorpromazine) and ethyl alcohol. Great differences appeared in the results, however. Mice anaesthetized with volatile anaesthetics which are easily removed by artificial respiration recovered postural reflexes earlier than those given the other drugs, although heart beats and breathing had not appeared sooner. Mice cooled under chloroform recovered rapidly but died within 3 days. Uncooled controls died either within a few minutes or within 3 days of chloroform anaesthesia. Temporary weakness or paralysis of the hind legs was less frequent in animals treated by agents known to produce vasodilatation. Since weakness of hind limbs rarely appeared after revival of animals cooled in closed vessels, mice were placed in jars in which the accumulation of CO_2 was prevented by the addition of soda-lime. In these experiments the numbers of complete revivals were fewer and there were increases in frequency of hind limb damage. When water vapour was removed by silica gel and CO_2 allowed to accumulate, the animals had fewer and less severe evidences of damage from the standard exposure to cold. The importance of CO_2 in the induction of and in resuscitation from severe hypothermia may be related to its effects upon the vascular system and upon oxygen requirements.

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Observations on an amine oxidase of mammalian plasma.

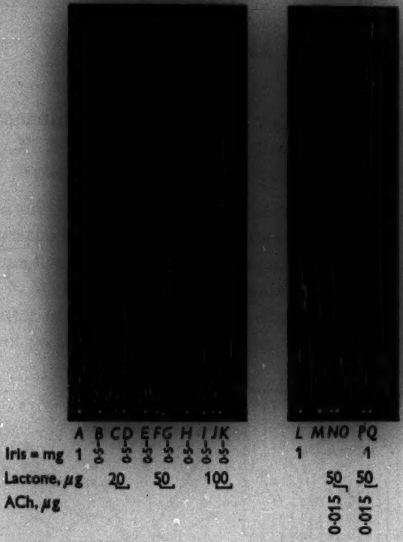
By H. Blaschko, Giovanna Ferro-Luzzi and Rosemary Hawes. *Department of Pharmacology, University of Oxford*

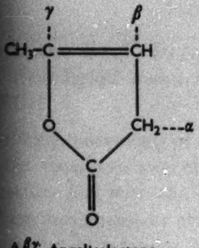
The unsaturated nature of irin and its interaction with lactones.

By N. Ambache. *Medical Research Council Institute of Ophthalmology, Judd St, London, W.C.* 1

1–2 % phenyl isocyanate, which reacts with OH groups, inactivates irin. Ether-purified irin residues (*a*) contain a lactonizing hydroxy-fatty acid (HFA) giving (1) an HFA red colour with *sym* diphenyl-carbazide, (2) a Fe hydroxamate absorbing at 540 mμ; (*b*) have an infra-red absorption at 1740 cm^{-1} consistent with a δ-lactone, either saturated or possibly $\beta\gamma$-unsaturated ($\Delta^{\beta\gamma}$)

but not $\Delta^{\alpha\beta}$- or enol-lactone (i.e. $\Delta^{\gamma\delta}$). The effect was therefore studied of some lactones on the unatropinized hamster colon. None of these $(0{\cdot}4–4 \times 10^{-5})$ contracted the muscle, but several potentiated or blocked irin contractions. Irin itself is unsaturated and is inactivated in 5–10 min by 0·0005 $KMnO_4$; and in 10–30 min by 0·005 M-IBr, which indicates that the essential double bond is not in a position *αβ* to carbonyl.





$\Delta^{\beta\gamma}$-Angelicalactone

Fig. 1

The saturated δ-dodecalactone (but not *β*-methyl-δ-hexanolactone) blocked irin transiently, as did the *αβ*-unsaturated lactones coumarin and (more durably) patulin.

βγ-unsaturated γ- or δ-lactones. $\Delta^{\beta\gamma}$ angelica-lactone (Fig. 1 arrows) potentiated the effect of watery iris extract (dots) but not of acetylcholine (asterisks). Laevulinic acid (Na), of which angelica- is the isomeric enol-lactone, did not potentiate. *β*-methyl-$\Delta^{\beta\gamma}$-δ-hexenolactone produced little or no effect, but *β*-methyl substitution is known to interfere with the reactivity of $\Delta^{\beta\gamma}$-lactones.

Role of the area postrema in the vomiting induced by nitrogen mustard and by X-radiation.

By H. L. Borison, E. D. Brand and R. K. Okkand. *University of Utah College of Medicine, Salt Lake City, Utah, U.S.A. and University of Virginia College of Medicine, Charlotteville, Virginia, U.S.A.*

PROCEEDINGS OF THE PHYSIOLOGICAL SOCIETY

NATIONAL HOSPITAL, QUEEN SQUARE, LONDON, W.C. 1

13–14 *December* 1957

Recording compensatory rolling of the eyes.

By T. Davies and P. A. Merton. *Medical Research Council Neurological Research Unit, National Hospital, Queen Square, London, W.C.* 1

In a previous demonstration (Merton, 1956) the rolling movements of the eye when the body is swung from side to side around the visual axis were observed through a low-power microscope. At the suggestion and with the help of Dr F. W. Campbell we now obtain a record of these movements by attaching a piece of birefringent mica to the eye and measuring its rotations by their effect on polarized infra-red light.

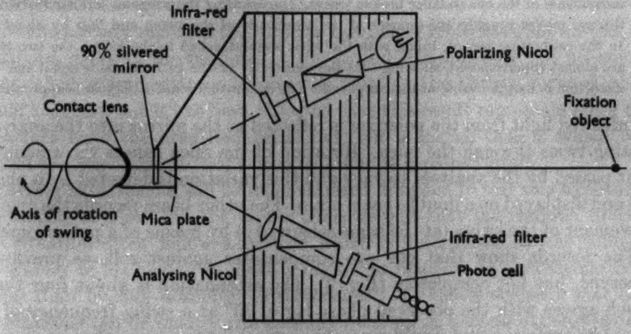

Infra-red filter
90% silvered mirror
Polarizing Nicol
Contact lens
Fixation object
Axis of rotation of swing
Mica plate
Infra-red filter
Analysing Nicol
Photo cell

Fig. 1. Diagram of the recording equipment. The light source is a 6 W car lamp, the photocell a Mullard 61 SV.

In ordinary ophthalmological practice contact lenses are designed to bear on the bulbar conjunctiva, standing clear of the cornea. Such lenses do not follow rolling of the eye, for in rolling the bulbar conjunctiva twists on the bulb as a stocking can be twisted on the leg. In some wearers the lenses, by an unknown mechanism, are sucked on to the cornea and become firmly adherent to it. For accurately recording rolling movements a small corneal lens taking advantage of this action was very kindly made for us by Mr F. Ridley of Moorfields. It carries the mica plate on a metal stalk 15 mm long. After half an hour it is so firmly stuck as to be troublesome to pull off, and to permit the eye to be moved about independently of the subject's will by manipulating the stalk.

The polarizer and analyser are mounted on a common frame which also carries a small mirror on an arm (Fig. 1). The subject's head being steadied by a dental grip so that his pupil is in the axis of rotation of the swing, the mirror is adjusted to lie just behind the mica plate but so that the mirror and arm do not foul the plate and stalk, or the subject's nose, when the swing is going. In order to allow the subject to see a distant fixation point on the axis of the swing the mirror is not completely silvered.

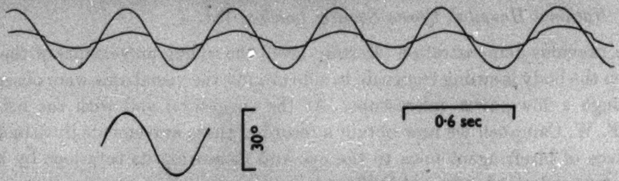


Fig. 2. Records of the excursion of the swing (large smooth sinusoidal trace) and of the rolling movements of the eye (smaller broken trace). Disregarding the irregular jerking movements the eye moves roughly one-quarter of the amplitude of the swing and lags by about 15°. In the calibration record, taken with the mica plate attached to the swing, the two traces are almost superimposed, showing that the sensitivity of the two records is equal and that distortion is inappreciable at this amplitude. The calibration scale of degrees is approximate.

Infra-red light from the polarizer is reflected at the mirror into the analyser, passing twice through the mica. Rotation of the mica causes the amount of light passed by the analyser to vary and this variation is detected by a photocell and displayed on a double-beam C.R.O. The other beam records the angular movement of the swing (at the same sensitivity) by means of a potentiometer.

The records show that the eye compensates against roll, as previously observed, but not completely (Fig. 2). The attenuation is about four times, which agrees with the observed phase lag of 15° at a swing frequency of 0·6 complete cycle/sec, and would give a time constant for decay of compensation after a single half swing of 1 sec (cf. Merton, 1956). Superimposed on the smooth movements are rapid saccadic rolling movements, more frequent when an object is being looked over by the eye, and tending to disappear when a point is intently fixated. A new observation is that these saccades mainly occur when the swing is moving most rapidly and not when the inclination is greatest.

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Interference between the responses of the human subject to two nearly simultaneous stimuli.

By A. Elithorn and A. M. Halliday.
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The fact that the reaction time to the second of two stimuli was found to be prolonged when this occurred closely following the first, has led elsewhere to the development of a concept of the human operator as a single channel mechanism. It is postulated that a 'decision process' occupies this mechanism for a finite time and that any second stimulus to which a response has to be made must wait until the first stimulus has been dealt with. A simple mathematical model has been proposed to predict the delays to be expected (Welford, 1952) and, with minor modifications, this has been accepted as fitting some further experimental findings (Davis, 1957).

The present experimental situation has been developed to put this hypothesis to a rigorous test. The subject sits facing a display with a central fixation point, which also acts as a warning light. Two clusters of neons provide the two stimuli, one in each half of the visual field. The time interval between the two stimuli is automatically selected from a predetermined series by a punched tape made up from random number tables. The responses are made by pressing two morse keys with the left and right hands. Since the response is made on the same side as the stimulus appears, the two responses are independent as regards their sensory and motor pathways.

Results already reported (Elithorn & Lawrence, 1955) have shown that the maximum delay in the response to the second stimulus does not necessarily occur when it immediately follows the first (as the theory outlined above would suggest), but it has been objected that this result represents not a true departure from the predicted behaviour, but a special case in which both stimuli are responded to, not separately, but as a paired group.

Further experiments will be demonstrated designed to test this hypothesis. The results of these and the earlier trials suggest that the reaction times in this situation often follow a pattern which is determined by the subject's expectancy of his having to make a particular response at a particular time, rather than by any absolute 'refractoriness'. Evidence suggests that such expectations can be readily and speedily built up in a given experiment and may be quickly changed when the range of stimuli is altered.

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A tachistoscope for the study of visual perception in homonymous half-fields.

By ELIZABETH WARRINGTON. *Psychological Laboratory, Institute of Neurology, Queen Square, London, W.C.* 1

This tachistoscope enables one to present visual stimuli to the whole visual field or to each half-field independently. When the apparatus is being used to test the homonymous half-fields of vision, simultaneous or successive stimuli can be exposed. In the later case the exposure time of each stimulus and the time interval between the two can be independently varied. The temporal order of the stimuli can be reversed. Exposure times range from 10 msec to 10 sec, and this range is graduated in steps of 10 msec. The minimum exposure time is below recognition threshold for a normal subject on this apparatus. The screen is viewed binocularly by the subject and subtends a visual angle of 8° either side of the fixation point.

An investigation of visual extinction (inattention) is in progress. It is hoped to assess the temporal relationships of the phenomena and in particular to relate the findings to the tachistoscopic threshold for each homonymous field.

Designed by R. L. Gregory of the Psychological Laboratory, Cambridge.

Apparatus for the study of visual size and shape constancy.

By MARIA WYKE. *Psychological Laboratory, Institute of Neurology, National Hospital, Queen Square, London, W.C.* 1

Successful adaptation of higher organisms to their environment depends, amongst other things, on their constancy of visual perception. The optical properties of the eye are inadequate to provide such perceptual constancy; therefore this function must involve central nervous mechanisms additional to those in the retinal projection system. Previous reports (Bender & Teuber, 1949; Critchley, 1950) have suggested that alterations in perception of the size and shape of objects may occur with cortical lesions.

Comparative experiments are being performed on normal and abnormal subjects to verify the relation between cortical lesions and changes in perceptual constancy. An attempt has also been made to determine whether such changes are more particularly associated with lesions in individual cortical sectors.

Two types of apparatus are being used to study this problem quantitatively, in terms of constancy of shape and constancy of size; and these are demonstrated.

Constancy of shape. The subject gazes into a tapered rectangle 2·5 m long, with a non-reflective internal surface. At the opposite end is an evenly-illuminated opal glass screen, in front of which two stands carry, side by side, the test objects. The stimulus objects are ellipses of constant major, but varying minor diameters. The comparison object is a circle, which the subject rotates by a remote control until it appears to be the same shape as the presented ellipse. The angle of rotation of the circle is read off from a scale, and compared with the true projection angle of the ellipse.

Constancy of size. The subject gazes into a rectangular, black-lined box 3·0 m long. The stimulus objects are presented at one side of the box at a distance of 1·0 m from the subject, and consist of illuminated triangles of different sizes. The comparison object is 3·0 m from the subject at the other side of the box, and is an illuminated triangle whose size can be remotely adjusted by the subject until it appears to be the same size as the test object. The size of the comparison object is then read off from a scale, and compared with the real size of the stimulus object.

The apparatus was constructed in the Department of Applied Physiology, Royal College of Surgeons of England, by Mr Steward.

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Immediate and chronic effects of phenol on nerve roots.

By P. W. Nathan, T. A. Sears and Marion C. Smith. *The National Hospital, Queen Square, London, W.C.* 1

The effect of chymotryptic digestion on caeruloplasmin.

By G. Curzon. *The National Hospital, Queen Square, London, W.C.* 1

Cerebral lipids in infants.

By J. N. Cumings. *The National Hospital, Queen Square, London, W.C.* 1

A pressurized cranial window.

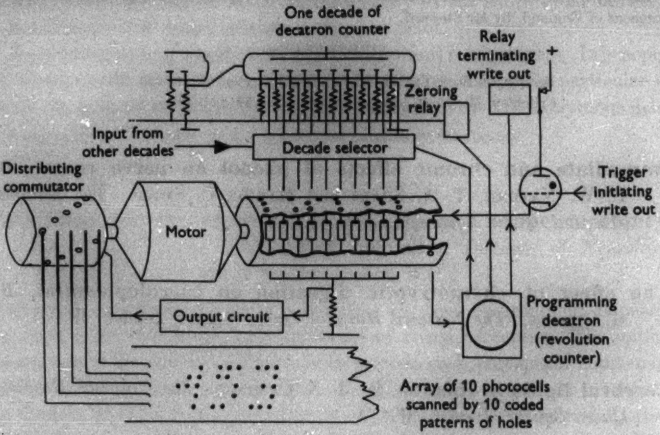
By R. Efron. *The National Hospital, Queen Square, London, W.C.* 1

A new high-speed printing counter.

By T. V. Edwards and A. M. Halliday. *Medical Research Council Neurological Research Unit, National Hospital, Queen Square, London, W.C.* 1

A device has been developed for recording numbers derived from electronic counting circuits (such as those employing decatrons or hard valves) which will operate at very much higher speeds than conventional printing counters which use registers with moving mechanical type. The numbers are inscribed on electrically sensitive paper (teledeltos) as a series of dots marked out by five electrical contacts, arranged side by side, which are repeatedly scanned by a single output circuit through a distributing commutator. On the same shaft as the commutator, and therefore rotating at the same rate, is a second cylinder of opaque material revolving round an array of ten miniature germanium junction photocells. Holes can be made in this cylinder corresponding to the dots required, and ten sets of such holes are chosen to give the dotted outline of each of the ten digits from 0 to 9, each set revolving over one of the photocells.

The desired digit is inscribed simply by feeding an appropriate voltage to the anode of the corresponding photocell, and this voltage can be derived directly from the output of the counting circuit (e.g. a decatron cathode).


One decade of decatron counter
Relay terminating write out
+
Zeroing relay
Input from other decades
Decade selector
Distributing commutator
Trigger initiating write out
Motor
Output circuit
Programming decatron (revolution counter)
Array of 10 photocells scanned by 10 coded patterns of holes

As only one of the photocells has a voltage on it at any one time (corresponding to the struck cathode) the ten photocells can have a common cathode load and drive a single output channel, the latter giving low impedance pulses suitable for recording.

The initiation and termination of the reading cycle, the scanning of successive decades of the counting unit and automatic zeroing following write-out are readily arranged for by a single programming decatron, driven once per revolution by a single photocell mounted alongside the other ten. This operates a suitable arrangement of diode gates (for decade selection) and high-speed relays (for resetting or terminating the read cycle after write-out). The voltage supply to the photocell driving the programming decatron is derived through a thyratron which is extinguished at the end of the write-out cycle and can be re-triggered externally to re-start it. As each revolution of the commutator corresponds to the writing-out time of one digit and the over-all programming is a simple multiple of it, the speed of write-out can be continuously varied (within the limits of frequency response of the circuit components) by varying the speed of the drive motor.

The highest speed of writing is also limited by the total number of dots required. From a matrix of possible dots with a minimum total of fifteen (arranged, in this case, when written out, in three vertical rows of five) all ten digits can be outlined with a difference of at least three dots distinguishing any two digits. The outline of the numbers is clearly and immediately recognizable even with this relatively small number of total dots. With an increase in the total number (necessarily accompanied by a corresponding sacrifice in the maximum speed of printing) a more detailed outline could, of course, be traced. A larger number of dots would also be required if it was desired to print letters as well as figures.

Sensory nerve conduction in patients with peripheral nerve lesions.

By R. W. GILLIATT and T. A. SEARS. *The National Hospital*, *Queen Square*, *London*, *W.C.* 1

An expansion of the push-pull principle applied to a 16-channel electroencephalograph.

By H. B. MORTON. *National Hospital*, *Queen Square*, *London*, *W.C.* 1

In conventional multi-channel electroencephalographs each pen-recorder is driven by its own push-pull amplifier, and the recent trend has been to increase the number of recorders (up to 30) without a corresponding increase in the number of electrodes (usually about 20); thus the number of input grids exceeds the total of electrodes and economy might be achieved by using a singled-sided amplifier for each electrode rather than a push-pull amplifier for each pen.

In the machine demonstrated the cathodes of all the electrode amplifiers are connected to a common high impedance, this arrangement being regarded as an expansion of the push-pull principle. Since this makes it necessary that all the amplifiers shall have the same sensitivity and frequency response characteristics the controls are reduced to three, for gain, time-constant and high-frequency response.

The three systems of recording, bipolar in push-pull pairs, unipolar with reference to a single common electrode, or unipolar with reference to all the electrodes in use, are available by selecting the appropriate pairings of electrode amplifiers; this selection is made at the amplifier outputs, at a signal level of volts, so that selection can be made during recording without amplifier blockage or other artifacts.

The machine also incorporates a flat 32 × 36 selection switch, very accessible for cleaning, and a device for instantaneous graphic representation of the electrode grouping in use.

A stereotactic method suitable for the conscious human subject.

By J. A. V. Bates. *Medical Research Council Neurological Research Unit, National Hospital, Queen Square, London, W.C.* 1

A multi-channel electronic averaging system.

By G. D. Dawson and J. R. Pitman. *Medical Research Council Neurological Research Unit, National Hospital, Queen Square, London, W.C.* 1

A 21-channel E.E.G. apparatus.

By J. A. V. Bates and J. D. Cooper. *Medical Research Council Neurological Research Unit, National Hospital, Queen Square, London, W.C.* 1

The reabsorption of urea by the rat kidney.

By J. A. Barclay and R. F. Crampton. *Department of Physiology, University of Birmingham*

Albino rats were given subcutaneous injections of inulin, and after an equilibration period of 45 min, the kidneys were removed. The urea and inulin contents of the morphological zones of the kidney were then determined. The significance of the inulin content of these zones has previously been described (Barclay & Crampton, 1957). The results for inulin and urea have been expressed as ratios of the amount of each in the tissue (mg/100 g initial wet weight) to the concentration in the plasma (mg/100 ml.); see Table 1.

Table 1. The tissue/plasma ratios of inulin and urea for zones 1 and 4 of the rat kidney. Mean ± s.e. with number of observations in parentheses

Zone	Inulin T/P	Urea T/P	
1	2·88 ± 0·43 (30)	3·12 ± 0·38 (31)	$t = 0{\cdot}45$, $n = 59$, $P > 0{\cdot}6$
4	9·69 ± 1·21 (18)	29·4 ± 2·39 (18)	$t = 7{\cdot}36$, $n = 34$, $P < 0{\cdot}001$

The ratios for urea and inulin were equal in zone 1, indicating a high degree of impermeability of the proximal convoluted tubule to urea. In zone 4 the ratio for urea was approximately three times that of inulin. Ullrich & Jarausch (1956) found large amounts of urea in the medulla of the dog kidney, and concluded that the cells in this zone were highly permeable to urea. This finding, together with the data presented above, indicate that the collecting tubules are the site of the major part of urea reabsorption. The reabsorption of water through the collecting tubules (Wirz, Hargitay & Kuhn, 1951) would be followed by the passive reabsorption of urea. The further possibility that urea reabsorption across the collecting tubules occurs due to its high concentration produced by non-reabsorption proximally is not excluded by these results.

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In vitro measurement of inulin space of slices of rat skeletal muscle.

By R. F. Crampton and C. T. G. Flear. *Department of Physiology, University of Birmingham, and Gonville and Caius College, University of Cambridge*

The inulin space of the rat diaphragm, as measured *in vitro* by Creese (1954), was 26·4 ± 0·63 (s.e.) ml./100 g tissue. The inulin space of the anterior tibial muscle, determined on whole muscles, was found to be 11·2 ± 0·35 ml./100 g wet weight, a value of the same order as that found by Boyle, Conway, Kane & O'Reilly (1941) for the frog sartorius (9·6 ml./100 g tissue), also determined on whole muscle. The chloride space for the anterior tibial muscle of the rat was 12·7 ± 0·37 nl./100 g. Using slices of anterior tibial muscle (approximately 0·3 mm thick), a value of 28·8 ± 1·13 ml./100 g was found; the differences between this value and those for whole muscle spaces for inulin and chloride were highly significant ($P < 0{\cdot}001$). These slices of muscle lost 23·5 ± 0·63 % of initial wet weight after the usual time of 40 min in 0·9 % saline containing 0·2 % inulin (alkali stable) and the solution became turbid. Slices cut transversely gave higher values for inulin space than those cut obliquely or longitudinally. The loss of weight and high space values seemed to be due to loss of protoplasm from the cut ends of muscle fibres and again of inulin. In an attempt to diminish these effects, muscle slices were cut longitudinally and the surfaces composed of cut ends of fibres were cauterized with a small wire loop at red heat. Loss of tissue water effected by cautery amounted to 4·9 ± 0·91 g/100 g tissue. Weight loss after 40 min in inulin solution was 8·8 ± 0·83 %, and the inulin space of the cauterized slices, corrected for evaporation of water, 15·5 ± 0·61 ml./100 g.

The conclusions drawn from the above findings are first, that the extracellular space of different muscles can vary appreciably, and secondly, that the extracellular space may be measured on muscles where the chloride content is not a reliable guide, where it is not desirable to give an intravenous infusion of inulin and where only small portions of whole muscles are available. Such would be the case of muscle biopsies from some patients with severe electrolyte disturbances.

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The weakness of the Hering–Breuer reflexes in man.

By R. Marshall and J. G. Widdicombe. *Department of Physiology and the Dunn Laboratory, St Bartholomew's Hospital Medical College, London, E.C.* 1

Christiansen & Haldane (1914) have investigated the Hering–Breuer reflexes in conscious man. In the light of more recent work their records suggest that the inflation reflex (an inhibition of breathing) is very feeble and the deflation reflex (an increase in inspiratory vigour, in respiratory rate or in both) is virtually absent. We have repeated their experiments with unanaesthetized man, and of ten subjects only one gave an inhibition of breathing on inflation of the lungs. The others usually increased both rate and depth of breathing. All subjects responded to deflation of the lungs by an increase in the depth of inspiration. Since it was possible that the reflexes were masked or distorted by cerebral influences, we have investigated the reflexes in four anaesthetized patients (without lung disease). Although both inflation and deflation reflexes were detectable in three of these four, they were many times weaker than in experimental animals. In dogs, cats, rabbits, guinea-pigs, rats and mice lung inflations with a transpulmonary pressure of 10 cm H_2O inhibited breathing for 45–90 sec, while in man the longest inhibition for a comparable pressure change was only 10 sec. The deflation reflex showed a similar but smaller difference. The human lung probably contains pulmonary stretch receptors (Larsell & Dow, 1933), but their action on the respiratory cycle must be relatively weak.

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A sensitive *in vitro* technique for the assay of thyrotrophic hormone.

By P. M. Bottari* and B. T. Donovan. *Department of Neuroendocrinology, Institute of Psychiatry, Denmark Hill, London, S.E.* 5

Studies upon the physiology of guinea-pig thyroid tissue cultured *in vitro* have revealed that thyrotrophic hormone (TSH) exerts a quantitative effect upon the release of ^{131}I labelled protein-bound iodine from the gland. This action has been utilized in the development of a new assay for the pituitary hormone.

Both lobes of the thyroid gland of a guinea-pig are cut into small pieces and the fragments divided among six test tubes for incubation in a nutrient medium containing ^{131}I. After 18 hr sodium iodide is added to the tubes to prevent recirculation of labelled iodine and a sample of the medium removed for measurement of its radioactivity. Comparison with control solutions then permits the calculation of the amount of ^{131}I picked up by the thyroid tissue.

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The fluid to be assayed is added and a second portion of incubating medium removed after 2 hr for estimation of radioactivity. The percentage release of labelled iodine may then be calculated from the change in ^{131}I concentration in the medium. A linear log–dose response curve is obtained from serial dilution of a standard preparation of TSH and spans a range of sensitivity between one-thousandth of a milliunit and 10 mu. of International Standard. At present a standard preparation of TSH is added to three tubes in order to draw a dose-response curve, and the remaining three tubes are used for the determination of the TSH content of the fluid added. Separated plasma or serum may be used without further manipulation and with this technique the blood of normal rabbits has been shown to contain about 15 mu. TSH/100 ml.

The current method has a number of advantages over that previously described (Bottari, 1956, 1957) in that it is less time-consuming, simpler, and more accurate.

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The velocity of combination of the first oxygen molecule with sheep haemoglobin.

By Q. H. Gibson and F. J. W. Roughton, *Departments of Biochemistry, University of Sheffield and Colloid Science, University of Cambridge*

Hartridge & Roughton's (1923, 1925) measurements of the rate of combination of O_2 with sheep haemoglobin were made before the development of Adair's (1925) intermediate compound hypothesis, and at a time when the dissociation curve of dilute haemoglobin solutions was believed to be hyperbolic. On the latter ground they interpreted their results in terms of the equation

$$\mathrm{d}[O_2Hb]/\mathrm{d}t = k'[O_2]\,[Hb] - k[O_2Hb].$$

Such an equation is not compatible with the intermediate compound hypothesis except during the earliest stages in which the only effective reaction is $Hb_4 + O_2 \underset{k_1}{\overset{k'_1}{\rightleftharpoons}} Hb_4O_2$, the concentration of higher intermediates, e.g. Hb_4O_4, Hb_4O_6, not yet being appreciable. The limiting value of Hartridge & Roughton's k' at zero time should be equivalent to $k'_1/4$, but since the observing instrument they used (the reversion spectroscope) was limited to determinations above 30% saturation, it is not easy to extrapolate their data to zero time. Photoelectronic observations are, in principle, applicable over the whole range of percentage saturation, and we have recently applied them to the rate of combination of sheep Hb solutions (3×10^{-6} equiv to 8×10^{-5} equiv, i.e. blood

1 in 2500 to blood 1 in 100) with dissolved O_2 (1×10^{-5} M to 1×10^{-4} M, corresponding to O_2 pressures of 5 to 50 mm. Hg) using both the stopped flow and moving flow methods for recording rapid reaction rates. Our limiting values for k' at zero time are from 1×10^{6} to $1{\cdot}8\times10^{6}$ M^{-1} sec^{-1} at pH 9·1. These figures are only one-third to one-half of the values for k' obtained by Hartridge & Roughton from the times of half combination at higher (e.g. 10^{-4} M) oxygen concentrations. In these circumstances the later and faster reactions of the intermediate series (e.g. $Hb_4O_2+O_2\rightarrow Hb_4O_4$) have a pronounced accelerating effect which accounts satisfactorily for the discrepancy. On the other hand, at low O_2 concentrations where the final equilibrium is reached at 10–20% O_2Hb, the values of k' as calculated from the half time are markedly less than the extrapolated values at zero time. This is due to the effect of the back reaction $Hb_4O_2\rightarrow Hb_4+O_2$ predominating over the accelerating effect of the later combination reactions $Hb_4O_2+O_2\rightarrow Hb_4O_4$, etc. Observations have also been made at pH 7·1, but were complicated by a tendency towards a biphasic approach to equilibrium, a phenomenon also found in lesser degree at pH 9·1, especially at low O_2 and low Hb concentrations.

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Visual discrimination following successive unilateral temporal excisions in monkeys.

By G. Ettlinger. *From the Psychological Laboratory, Institute of Neurology, University of London*

It has been known since the classical investigation of Klüver & Bucy (1938) that bilateral temporal excisions in monkeys are followed by a severe disorder of visual perception. Despite intensive experimentation the nature of this disturbance is not yet understood. Since unilateral temporal lesions fail to give rise to any visual loss it has been assumed that the deficit is more closely akin to an agnosia than to a field defect.

The effects of various lesions on the ability to learn and retain a simple pattern discrimination (square or cross) have been investigated on twelve monkeys. The lesions consisted of various combinations of the following carried out at one or more operations: (*a*) section of the left optic tract, (*b*) a left temporal excision, (*c*) a right temporal excision, (*d*) section of the corpus callosum. Training was carried out either before or after the first operation and quantitative testing at every stage. It was found that no impairment followed lesions (*a*) and (*b*) or (*a*) and (*d*), whereas moderate but consistent impairment followed lesions (*a*) and (*c*).

Animals with pre-existing left tract sections and left temporal excisions became severely impaired, as expected, following excision of the right temporal region. Animals with pre-existing left tract sections and right temporal excisions showed further moderate impairment following excision of the left temporal region or section of the corpus callosum. Animals with pre-existing left tract and callosal sections became more severely impaired following excision of the right temporal region than did those animals having a left tract section combined with a right temporal excision but with intact corpus callosum.

These findings suggest that in the monkey each temporal region is related preferentially, but not exclusively, to input from the contralateral half visual fields. Further, following excision of the temporal region on one side, the corpus callosum is critically implicated in the interaction of the remaining temporal region with the input from the ipsilateral half visual field.

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The role of CO_2 in the changes in forearm vascular resistance with anoxia. By J. E. Black and I. C. Roddie. *Department of Physiology, The Queen's University of Belfast.*

Normal subjects were made to breathe 5–10% O_2 in N_2 for 2–6 min. Forearm blood flow was measured by venous occlusion plethysmography, brachial arterial pressure by capacitance manometry, antecubital venous pressure by saline manometry and respiratory movement by two stethographs, one round the chest and one round the abdomen connected to a single volume recorder. Anoxia caused a large increase in heart rate, forearm blood flow and ventilation. The arterial pressure was little changed and vascular resistance in the forearm, calculated by dividing mean pressure by mean flow, was found to be greatly reduced. A similar decrease in vascular resistance was seen in the nerve-blocked forearm suggesting that a humoral rather than a nervous mechanism was responsible for the vasodilatation.

These vascular changes in the forearm with anoxia are similar to those which occur during voluntary hyperventilation (Roddie, Shepherd & Whelan, 1957). With voluntary hyperventilation humoral vasodilatation occurs in the forearm and is due to washing out of CO_2 from the lungs, since it does not occur if CO_2 is added to the inspired air in an amount calculated to keep the alveolar CO_2 constant. Since the hyperventilation with anoxia causes washing out of CO_2 it was thought possible that the vascular changes with anoxia might result more from the resultant hypocapnia than from the lack of oxygen *per se*. To test this hypothesis subjects were made to breathe mixtures of 5–10% O_2

in N_2 to which an amount of CO_2 was added which was calculated to keep the alveolar CO_2 nearly constant. When this condition was fulfilled it was found that the increase in blood flow in the normal and nerve-blocked forearms was greatly reduced, as was the increase in heart rate. Further, there was a rise in systolic, diastolic and mean arterial blood pressure. The increases in forearm blood flow could now be explained by the increases in perfusion pressure; vascular resistance was not altered to an extent which could be appreciated using the present methods. It is concluded that peripheral resistance in the forearm is not increased during the pressor response to anoxia. In this respect the results are similar to those seen during the pressor response to common carotid artery compression in man (Roddie & Shepherd, 1957).

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The effect of continuous positive and negative pressure breathing upon the capacity and resistance blood vessels of the human forearm and hand.

By D. A. Blair, W. E. Glover and B. S. L. Kidd. *Department of Physiology, The Queen's University of Belfast*

Subjects breathed from a small well-ventilated vessel in which steady pressures, ranging from 15 mm Hg above to 15 mm Hg below atmospheric pressure, were maintained by a large displacement (vacuum cleaner) pump. The forearm or hand was supported above heart level, and was enclosed in a water-filled, mechanically stirred plethysmograph.

The behaviour of the capacity vessels was deduced by measuring the volume increase brought about by graded venous congestion (Glover, Greenfield, Kidd & Whelan, 1957), and of the resistance vessels from measurements of blood flow by venous occlusion plethysmography.

The amount of blood which could be collected either in the forearm or hand on the application of a congesting cuff was not altered by breathing at these pressures.

Although there was a variation in response from subject to subject, the forearm blood flow was usually decreased, sometimes by as much as 50%, during positive pressure breathing; during negative pressure breathing it was usually increased, sometimes by as much as 40%. These changes in blood flow were due mainly to changes in the calibre of the resistance vessels, since there was little change in arterial pressure during the pressure breathing. As the changes in flow were abolished by nerve block the changes in calibre were mediated through nerves. No consistent change in hand blood flow was observed during pressure breathing.

It is suggested that the changes in forearm blood flow are caused by reflex responses of the resistance blood vessels to stimulation of receptors in the low-pressure blood vessels of the chest (Roddie, Shepherd & Whelan, 1957), and that the capacity blood vessels do not participate in this reflex response.

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Blood flow requirements of the calf muscles after walking and running.

By J. E. Black. *Department of Physiology, The Queen's University of Belfast*

Subjects either walked or ran 130 yards round a track at speeds varying from 1 to 8 m.p.h. with a light celluloid plethysmograph (Shepherd, 1948) in place on the calf. They then quickly lay down, and measurements of blood flow were made at 15 sec intervals by venous occlusion plethysmography and the total excess flow above the pre-exercise level was calculated. The first flow (peak flow) could be measured within 5 sec of the end of exercise.

The peak flow was increased by even the slowest walking to several times the resting flow in a recumbent subject. It increased progressively with speed of walking up to about 3·5 m.p.h., at which speed it reached about 35 ml./100 ml./min. Further increase of speed, up to 8 m.p.h., caused little further increase in peak flow, the highest value observed being 44 ml./100 ml./min. At speeds up to about 3·5 m.p.h. a total of less than 10 ml. of extra blood, over and above resting blood flow, passed through each 100 ml. of calf after the exercise. This quantity did not vary much with speed between 1 and 3·5 m.p.h. At speeds above 3·5 m.p.h., the amount of extra blood increased with the speed of walking or running, and amounted to as much as 40 ml./100 ml. after running at 8 m.p.h.

It is concluded that during walking at speeds of up to 3·5 m.p.h. the muscle blood vessels are able to satisfy the muscle blood flow needs fairly adequately during the actual period of exercise. At speeds greater than this, peak blood flow is not further increased and the vessels become progressively less able to satisfy the flow requirements during the exercise so that a progressively greater volume flow of blood must pass through the limb after the exercise is over.

(130 yards = 119 m; 1 mile = 1·6 km; 3·5 miles = 5·6 km; 8 miles = 12·9 km).

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The local effect of carbon dioxide on the blood vessels of the human skin. By A. Diji and A. D. M. Greenfield. *Department of Physiology, The Queen's University of Belfast*

Although carbon dioxide is generally presumed to have a local vasodilator action on blood vessels there is little direct evidence that this is so in the human subject.

Subcutaneous injections into the human forearm of 30 ml. of pure CO_2, or of mixtures of CO_2 with nitrous oxide containing as little as 12·5% of CO_2, caused a widespread and significant rise in temperature of, but only a slight reddening of, the skin. Nitrous oxide alone, cyclopropane and air similarly injected were either without effect, or caused a much smaller and more localized rise in skin temperature. From this it is concluded that the rise in temperature on injecting CO_2 mixtures subcutaneously is mainly due to a specific vasodilator action of the CO_2 affecting particularly the resistance blood vessels.

The rise in skin temperature was as large and more widespread following 30 ml. of each of the mixtures containing 12·5% or more of CO_2 than following the subcutaneous injection of 10 μg of histamine acid phosphate; the reddening of the skin was much less with the CO_2 than with the histamine.

Adaptation to localized warming and cooling. By E. M. Glaser*, M. S. Hall and G. C. Whittow†. *Department of Physiology, The London Hospital Medical College, London, E.1*

Previous experiments have shown that repeated immersions of a hand in water at 4° C are followed after about 9 days by a diminution of the pain and of the rise in blood pressure and heart rate which normally accompanies such immersions, this adaptation being confined to the hand which had been repeatedly immersed (Glaser & Whittow, 1957). Preliminary tests showed that immersion of a hand at 47° C is also accompanied by pain and by a rise in blood pressure and heart rate, and that these responses also diminish or disappear after about 9 days if the same hand is repeatedly immersed each day.

Three subjects immersed the same hand six times daily for 60 sec at 47° C and six times daily for 60 sec at 4° C, the hand being in water at 31·5° C for 30 min before each series of six immersions and for 60 sec between identical immersions. Within 9–15 days the pain and the rise of blood pressure and heart rate diminished or disappeared both during hot and cold immersions. On the first day of the experiment the mean systolic blood pressure rose by 16 mm of mercury during immersions at 47° C and by 32 mm during immersions at

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4° C, while the mean diastolic blood pressure rose by 8 mm at 47° C and 28 mm at 4° C. At the end of the experiment the mean change of the systolic blood pressure was 0 at 47° C and +3 mm at 4° C, while that of the diastolic pressure was −2 mm at 47° C and +6 mm at 4° C. Single immersions of the opposite hand at the beginning and end of the experiment gave responses which resembled those obtained at the beginning of the experiment from the hand which was to be adapted.

The skin temperatures during immersions and the heat dissipation in a calorimeter at 31° C were the same in the adapted and unadapted hands. Iontophoresis of an antihistamine substance into the adapted hand caused no consistent modification of adaptation. Immersion of the adapted hand of one subject at 1° C for 10 min was accompanied after the first 3 min by severe pain and followed by slight tissue damage, there being no difference in this respect between the adapted and the unadapted hand. Localized changes in the blood vessels, changes in the production of histamine, or changes in the pain receptors are thus unlikely to have caused the adaptations observed. Simultaneous adaptation to localized warming and cooling resembles, however, experimental acclimatization to heat and cold (Glaser, 1949), and it appears to confirm the suggestion that such adaptations may be brought about by the brain (Glaser & Whittow, 1957).

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Medullary potentials following stimulation of the orbital cortex.

By P. P. Newman and J. H. Wolstencroft. *Department of Physiology, School of Medicine, University of Leeds*

Newman & Wolstencroft (1956, 1957) reported that in cats under sodium pentobarbital anaesthesia a fall in arterial blood pressure was produced by heating the carotid blood or by localized heating of the medulla with high-frequency currents. This response was inhibited by electrical stimulation of the posterior orbital gyrus.

Further experiments have been undertaken to demonstrate pathways between the orbital cortex and the medulla. In cats anaesthetized with sodium pentobarbital (45 mg/kg) or Dial–urethane (0·7 ml./kg) the surface of the orbital cortex was stimulated through bipolar silver electrodes. The ventral aspect of the pons and medulla was exposed for systematic exploration of cortically induced potentials using bipolar or concentric needle electrodes. With single shocks a variety of responses was obtained according to the position of the recording electrodes. Large, clear-cut responses appeared most frequently with the recording electrode in the ipsilateral upper medulla, close to the mid line and a few mm below the surface. Withdrawing the electrode towards the surface reduced the amplitude of the potential or reversed its polarity. The potential usually took the form of an initial monophasic response of about 2 msec duration and a latency of 1–3 msec, followed by smaller second and third deflexions which could be either positive or negative from the base line. The amplitude and number of the deflexions diminished as recordings were taken from points rostral and caudal to the upper part of the medulla. No evoked potentials could be detected in the region of the tegmentum and upper pons.

In a second series of experiments the effect of orbital strychninization was observed. Cortical spiking followed the application of strychnine to the orbital surface after an interval of one minute. Corresponding potentials in the form of low-voltage spikes or waves could be detected in the medulla. They were either synchronous with the cortical discharges or followed them with latencies up to 40 msec.

These experiments point to the existence of descending pathways from the orbital cortex to the ipsilateral medulla through which medullary activity might be influenced. The fact that strychnine applied to the cortex may elicit spikes in the medulla suggests that direct pathways are involved.

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The increase in alkaline phosphatase activity of the duodenum and decrease in absorption of antibodies by the gut induced in young rats by deoxycorticosterone acetate.

By R. Halliday. *A.R.C. Unit of Embryology, University College of North Wales, Bangor*

The suckling rat is able to absorb antibodies readily from immune milk or serum administered by mouth at any age up to 18 days. This ability afterwards declines and is lost completely by 21 days (Halliday, 1955*a*, *b*). The mouse resembles the rat in having a long post-natal period of antibody absorption (Ehrlich, 1892), but we have now shown that this period is shorter than in the rat, absorption declining after 13 days and being lost by 17 days.

It has been demonstrated (Moog, 1951) that there is a very marked increase in the alkaline phosphatase activity of the duodenal lining of the young mouse between 13 and 18 days of age. A precocious increase in the level of activity of this enzyme could be induced by the injection into 9- or 10-day-old mice of large doses of various steroid hormones, of which cortisone acetate was one of the more effective, and deoxycorticosterone acetate much less effective (Moog, 1955).

Our observation, that in the mouse the period of loss of absorptive ability by the gut coincided with the period of rise in alkaline phosphatase activity of the duodenal lining, suggested the possibility that the two processes might be related. Accordingly, rats aged 9 or 12 days were injected with 1–5 mg of deoxycorticosterone acetate, and their ability to absorb homologous antibodies tested after 3 days. It was found that this treatment resulted in a decline by at least two or three dilutions in the amount of antibody absorbed from a single dose of immune rat serum, and that in one-third of the animals absorption was stopped entirely 6 days before this normally occurs. The administration of 5 mg of deoxycorticosterone acetate by mouth had a precisely similar effect, causing in all cases a marked lowering in the absorption of homologous antibodies.

Determinations of the alkaline phosphatase levels of the duodenal entoderm of the young rat showed an increase in activity resembling that in the mouse, but occurring between 18 and 23 days of age. It is apparent, therefore, that in the rat also, a rise in the activity of this enzyme in the duodenal lining coincides with a decline in the ability to absorb antibodies from the gut. The injection, or oral administration, of 5 mg of deoxycorticosterone acetate into 12-day-old rats induced a rise in the alkaline phosphatase level of the duodenal entoderm to near the adult level.

Thus, the administration of deoxycorticosterone acetate by mouth or parenterally to young rats induces both a premature reduction or termination of the absorption of antibodies by the gut, and a premature increase in the alkaline phosphatase level of the duodenal entoderm.

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Excitatory effects of vagal stimulation on cooled atria and their relation to adrenaline.

By J. H. Burn and M. J. Rand. *Department of Pharmacology, University of Oxford*

We have previously described experiments with McEwen's (1956) preparation, which consists of the atria of the rabbit heart with the vagus nerves attached. The preparation is suspended in a bath and when the temperature is reduced below 20° C, the contractions cease. Stimulation of the vagi causes the contractions to begin again (Burn & Rand, 1957). It was possible that this excitatory effect of vagal stimulation was due to the release of adrenaline or noradrenaline. The following results exclude this possibility:

(1) ACh in a concentration 10^{-7} g/ml. will start the contractions of cooled atria (Marshall & Vaughan Williams, 1956).

(2) Atropine (10^{-7} g/ml.) prevents vagal stimulation from starting contractions, but in the presence of atropine adrenaline (10^{-7} g/ml.) will start contractions.

(3) The atria from cats behave like atria from rabbits. We removed both stellate ganglia and both superior cervical ganglia from four cats, and allowed time for degeneration of the sympathetic fibres. We found that when the atria were cooled until the beat was arrested, stimulation of the vagi caused the beats to begin again, and this effect was abolished by atropine.

(4) Bertler, Carlsson & Rosengren (1956) have shown that when rabbits are given reserpine (5 mg/kg intravenously) the hearts are found to have lost all noradrenaline and adrenaline after 16 hr. We administered 1·5 mg/kg intraperitoneally, followed by 5 mg/kg intravenously 24 hr later. After a further 16 hr, we set up the atria and by cooling the bath arrested the beat. Vagal stimulation then started the contractions. The contractions began in two experiments with two single pulses of 0·15 msec duration at 1 sec interval. The contractions having continued for some minutes were then arrested by one single pulse.

(5) The contractions of cooled atria occurring at irregular intervals of about 30 sec were made rapid, regular and stronger by the addition of muscarine (10^{-8} g/ml.) to the bath.

These observations indicate that the excitatory action of vagal stimulation is due to ACh and not to adrenaline or noradrenaline, and that the effect of ACh is muscarine-like; it is not an effect on ganglia.

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Effect of oestradiol, progesterone and relaxin on the physical properties of the uterine cervix of the rat.

By Beulah M. Cullen and R. D. Harkness. *Department of Physiology, University College London*

The experiments were done on the excised cervix suspended in Locke's solution at 22° C, and stretched between two parallel steel rods 0·6 mm in diameter, one through each canal (Harkness & Harkness, 1956). The force pulling apart the rods was increased stepwise by 25 g every 15 sec until the tissue ruptured. Breaking stress was related to the quantity of collagen present. A calculation, based on the assumption that the collagen was evenly distributed round the rods, gave an average figure of collagen of 0·5–1 kg/mm^2 cross-section. When allowance was made for variation in breaking stress, the cervices were found to differ principally in the height of their length–tension curves, which were all of similar shape. The inner circumference (2 × distance between rods plus circumference of one rod) at half the breaking stress was taken as a measure of length.

Preliminary experiments with progesterone (10 mg/day for up to 9 days subcutaneously in oil) and oestradiol (5 μg oestradiol cyclopentyl-propionate Depo-Oestradiol, Upjohn) in oil, given in a single dose to previously spayed virgin rats, showed that both could increase the inner circumference, though the effects were small. Relaxin* (75–300 units in suspension in 5% beeswax in peanut oil, Kroc & Steinetz, personal communication) given three days after a priming dose of oestradiol (5 μg) gave a small increase above that due to oestradiol alone in cervices examined 24 and 48 hr later. A more prolonged factorial experiment lasting 9 days in which progesterone (10 mg/day), oestradiol (5 μg on day 0), and relaxin (75 units, days 3, 5 and 7) were given in all possible combinations showed much larger effects. The greatest increase in the circumference of the cervix was found with all three combined. Values (mean ± S.E., 3 rats per group) for the circumference were: untreated 4·01 ± 0·20, R 4·50 ± 0·21, P 4·22 ± 0·34, O 5·90 ± 0·57, RP 4·47 ± 0·26, OP 6·81 ± 0·50, OR 8·78 ± 1·12, ROP 11·30 ± 0·37 mm. The effectiveness of relaxin suggests that it is concerned in the relaxation of the uterine cervix as well as of the pelvic ligaments in pregnancy.

* The preparation of relaxin used was Releasin, kindly provided by Dr R. L. Kroc of the Warner-Chilcott Laboratories.

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Anaphylactic shock in the albino rat. By R. K. Sanyal* and G. B. West.

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In 1952, Malkiel & Hargis reported that in the rat *Haemophilus pertussis* vaccine aggravates anaphylactic shock induced by horse serum. We have now confirmed this finding and noted similar results with fresh egg white. In anaphylactic shock in the rat the organ chiefly affected is the small intestine where oedema, haemorrhage and eosinophil infiltration occur in the submucosa and there is oedema and desquamation of the mucosa. Similar lesions can be found in isolated sensitized intestines on perfusion with antigen.

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The potentiating action of *H. pertussis* vaccine on the anaphylactic reaction in the rat is not due to an increased formation of antibody, nor is it due to an increased release of histamine and 5-hydroxytryptamine (5-HT) from the tissues. Further, the histamine and 5-HT contents of the tissues, the urinary histamine excretion under basal conditions and conditions of histamine load, and the histaminase activity of intestinal homogenates, are similarly unaltered. Such animals, however, are less resistant to exogenous histamine and 5-HT. Mepyramine, which partially protects animals pretreated with *H. pertussis* vaccine from lethal doses of histamine, fails to modify the anaphylactic shock. In a similar manner, brom-lysergic acid diethylamide (BOL 148) protects such animals from lethal doses of 5-HT but does not alter the anaphylactic reaction. Anaphylactic shock is also not altered by previous depletion of tissue histamine or 5-HT using polymyxin B or reserpine respectively (Parratt & West, 1957), or by previous X-irradiation (Eisen, Ellis & Wilson, 1956).

It is concluded that (*a*) *H. pertussis* vaccine aggravates anaphylactic shock in the rat, (*b*) the target organ is the small intestine, and (*c*) histamine and 5-HT do not play major roles in the reaction.

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Changes in the liver during the induction of pregnancy toxaemia in sheep.

By H. B. Parry and Heather J. Shelley. *Nuffield Institute for Medical Research, University of Oxford*

Fatty infiltration of the liver is well known to occur in sheep dying of pregnancy toxaemia (Groenewald, Graf & Clark, 1941), and in pregnant sheep subjected to a sudden reduction in food intake (Snook, 1939; Ferguson, 1954), a procedure which can be used to induce the disease experimentally (Parry, 1954). Sheep suffering from the disease may recover if parturition occurs.

Seventeen pregnant and five non-pregnant ewes were transferred from good pasture supplemented with concentrates and hay, to individual indoor pens with a maximum daily intake of 150–200 g concentrates, oat straw or hay. Liver biopsy samples were taken at 2- to 3-day intervals under local anaesthesia; blood samples were taken twice daily. Histological examination of the liver samples confirmed the results of chemical analysis. Nine ewes lambed during the course of the experiment; three of these had stillborn lambs which had died *in utero* 2–3 days earlier.

Table 1 shows that, whereas inanition was slow to affect the non-pregnant ewes, a rapid depletion of carbohydrate and the accumulation of large amounts of fat occurred in the livers of the pregnant ewes. These changes were paralleled by a fall in the blood (total reducing) sugar and a rise in blood ketones and were independent of the stage of gestation (111–143 days at the start of the experiment) and the foetal load (3 ewes carried single lambs, 13 twins and 1 triplets).

Table 1. The effect of inanition on the liver carbohydrate and neutral fat, blood sugar and ketone levels of pregnant and non-pregnant ewes

	Non-pregnant				Pregnant				
Period of inanition (days)...	0	7	14	18	0	2	7	14	21
Liver carbohydrate	40·7 (4)	64·2 (3)	40·1 (3)	32·0 (4)	43·2 (16)	6·2 (11)	11·9 (12)	11·9 (4)	11·8 (1)
Blood sugar	36·6 (4)	25·7 (4)	27·8 (4)	25·3 (4)	39·2 (12)	22·1 (12)	19·3 (9)	19·4 (3)	23·5 (1)
Liver fat	69·9 (4)	95·6 (3)	128·7 (3)	134·5 (2)	24·1 (14)	84·1 (9)	219·2 (12)	431·0 (2)	445·0 (2)
Blood total ketones	3·6 (4)	4·2 (4)	3·0 (4)	3·0 (4)	3·8 (15)	7·8 (14)	13·6 (12)	13·8 (4)	22·3 (2)

Liver carbohydrate and fat are expressed as mg/g lipid-free moist tissue, blood sugar and ketones as mg glucose or acetone per 100 ml. blood. Number of observations in parentheses.

Clinical signs of toxaemia in eight ewes were not related to the liver and blood constituents, which returned to normal levels following parturition or foetal death, even when no food was eaten.

This work was supported by a grant from the Nuffield Foundation.

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The frequency spectrum of the muscular activity of shivering in the anaesthetized cat.

By O. C. J. Lippold, J. W. T. Redfearn & J. Vučo. *Department of Physiology, University College London*

A normal muscular contraction is not perfectly smooth, and analysis of a myographic tension recording shows that there is a regular rhythmical component having a frequency about 9 c/s in the human and about 15 c/s in the cat. The mechanism of this rhythm seems to be based upon the stretch reflex servo-loop which has a tendency to self-oscillation at these frequencies (Lippold, Redfearn & Vučo, 1957).

There is, in addition, an irregular component in the frequency spectrum of normal muscular activity which does not depend upon the stretch reflex because it is still present after deafferentation of a muscle.

The muscular activity associated with shivering, both in man and in the anaesthetized cat, does not differ qualitatively from normal activity with regard to its frequency spectrum. In normal shivering muscle the rhythmical component is present but the random component is exaggerated; deafferentation has the effect of removing the rhythmical tremor, while the irregular tremor is maintained.

Birzis & Hemingway (1957) have shown that there is a pathway, which originates in the posterior part of the hypothalamus, concerned with the production of shivering in muscle. They have recorded from this tract impulses which are responsible for shivering, apparently in the form of a steady stream; they could detect no marked irregularity in the discharge which could account for the movements observed in the shivering muscles.

Part of the muscular activity of shivering must depend on the stretch reflex. The activity persisting after deafferentation of a muscle could be accounted for by the occurrence of some form of synchronization (or other modification of the descending discharge) at a spinal level.

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Adaptation to intermittent fasting in normal and denervated muscle.

By E. Gutmann & Gerta Vrbová. *Institute of Physiology, Czechoslovak Academy of Sciences, Prague*

In rats which have been submitted to a 6-week period of intermittent fasting, consisting successively of 3 days fasting and 1 day feeding, skeletal muscles examined during the final period of feeding show a considerable increase in their ability to synthesize glycogen and protein.

The increase in glycogen synthesis no longer occurs in muscles which have been denervated before the intermittent fasting. It is not affected, however, when denervation is performed after the intermittent fasting. The increase in protein synthesis is likewise abolished in muscles which have been denervated before the intermittent fasting. In these muscles there is only the early, transient and small increase in protein synthesis which also occurs without a long period of intermittent fasting.

We consider that the large increase of glycogen and protein synthesis which occurs after intermittent fasting is an adaptive reaction and that the nervous system is essential for its establishment.

The excitation of regenerating nerves by acetylcholine. By J. Diamond.

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It has been suggested (Diamond, 1955) that the sensitivity of receptor nerve endings to acetylcholine might be related to their probable specialization for the production of receptor potentials. If, however, this sensitivity were an independent characteristic of the 'exposed' terminal membrane, then regenerating nerve terminals, which are structurally similar, might show similar properties. This possibility has been investigated. Close arterial injections were made into the mid-calf region of sural nerves in rabbits anaesthetized with urethane. In the normal nerve, impulses were excited mechanically by the pressure of the injection, and by 0·2–0·4 ml. acetylcholine 10^{-5}–10^{-4} g/ml.; these impulses could be detected in the nerve central to the region perfused, but not peripherally; they appeared to be initiated in fibres which terminate in the nerve, its supporting tissue or the adjacent artery. Impulses excited by an injection of KCl were conducted both centrally and peripherally.

Similar experiments were done on rabbits whose sural nerves had been crushed or cut, under aseptic conditions, from a few hours to a few months previously. In these animals the injection of 0·2 ml. or more of acetylcholine or nicotine (10^{-4} g/ml.) always excited impulses which were conducted centrally but not peripherally. Unlike the response evoked by acetylcholine in normal nerve, that in regenerating fibres always outlasted the injection (which normally took less than 1 sec) by many seconds, and sometimes occurred when there was no response excited mechanically by the injection. The response to acetylcholine or nicotine was greatest when most of the regenerating fibres had grown to the region of the nerve which in these experiments was known to be the most efficiently perfused. In the normal nerve responses to acetylcholine comparable in magnitude to those obtained in regenerating nerves were produced only when the injected drug was allowed to reach skin innervated by the sural. In most experiments indian ink was injected to reveal the approximate extent of the perfusion.

The effects of acetylcholine on regenerating nerves were not secondary to vasomotor changes, being unaffected by atropine, paralleled by nicotine, blocked by hexamethonium and not mimicked by adrenaline, noradrenaline or $NaNO_2$; nor were the effects related to skeletal muscle activity. The most convincing responses occurred in experiments made 3 or more days after the nerve lesion was produced; other experiments suggested that some fibres became sensitive to acetylcholine within 12 hr. Nerve sprouts may appear within 12 hr after crushing (Perroncito, 1905, confirmed and quoted by Cajal, 1928). It seems likely that acetylcholine and nicotine act on the growing terminals. Experiments are in progress to determine whether regenerating motor fibres show similar properties.

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Excitation and conduction in smooth muscle.

By Edith Bülbring, G. Burnstock and Mollie E. Holman. *Department of Pharmacology, University of Oxford*

Excitability and conduction in rhythmically active smooth muscle (the taenia coli of the guinea-pig) were studied with external electrodes using the sucrose-gap technique (Burnstock & Straub, 1957) and recording in oil with wick electrodes. Single stimuli had no effect or caused only a brief disturbance of the spontaneous discharge. Repetitive stimulation, however, at a frequency approximating the spontaneous rate, set up responses of constant magnitude and latency. All irregular activity was abolished and the muscle could be 'driven' for periods of several hours. In preparations which were not spontaneously active a single stimulus produced a response which was identical with those recorded from driven preparations. The threshold for excitation was highly variable. Spikes were initiated at the cathode, their latency was directly proportional to the distance from the stimulating electrodes and they were conducted over many cell lengths without decrement. Conduction velocity varied from 6·7 to 8·8 cm/sec at 38° C. Both excitability and conduction were normal when nervous participation was excluded by (1) applying atropine 10^{-5}, (2) by using ganglion-free strips of taenia, (3) by using preparations which had been kept at room temperature for 27 hr.

Using intracellular recording and stimulating with a micro-electrode of relatively low resistance (10–20 MΩ) responses could only be recorded in another cell at distances of less than 0·5 mm. At greater distances an indirect influence was apparent, i.e. the rate but not the latencies of the responses were correlated with the stimuli.

The interaction between neighbouring cells was also studied during spontaneous activity. If two cells, within the same bundle and less than 1·0 mm apart, were impaled simultaneously, the spikes did not necessarily coincide but a spike in one cell was frequently associated with a small graded potential in the second cell.

Our experiments suggest that under normal conditions the spread of excitation is limited probably by locally initiated activity to a few cell lengths. If the spontaneous activity is absent or controlled by repetitive stimulation, conduction is possible over distances of at least 2 cm (longest length studied). Our results are consistent with the view put forward by Bozler (1941) and Prosser & Sperelakis (1956) that conduction in smooth muscle may take place by electrical transmission from cell to cell.

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The range of normal conduction velocity in motor fibres to the small muscles of the hand and foot.

By R. W. GILLIATT, T. A. SEARS and P. K. THOMAS. *The National Hospital, Queen Square, London, W.C.* 1

The action of procaine on fibres of different calibre in spinal roots of the cat.

By P. W. NATHAN and T. A. SEARS. *The National Hospital, Queen Square, London, W.C.* 1

This work attempts to analyse certain of the factors giving rise to the differential blocking of nerve fibres by local anaesthetics. The conduction velocities of component waves in the compound action potentials recorded from the dorsal and ventral roots were determined by stimulating the sciatic nerve or its branches at two different points. C-fibre compound action potentials were recorded from an isolated root preparation. Procaine was applied to the roots, either by flooding the laminectomy trough or by filling a small chamber through which the root passed. In these experiments the absence of a diffusion barrier, the perineurium, allows the full concentration of the applied anaesthetic to act immediately on the root.

As the block produced by 1% procaine develops, the amplitude of the α-wave (in either a dorsal or ventral root) diminishes without any significant lengthening of its latency (measured to peak), signifying that little slowing of conduction velocity occurs before block. We assume therefore that the height of the action potential at fixed latencies following the stimulus (corresponding to the principal elevations in the control record) is proportional to the number of fibres conducting at that velocity. Measured thus it was always found that a greater proportion of slowly conducting fibres (γ-δ-range) was blocked before rapidly conducting fibres (α-range).

The greatest degree of differential blocking was seen with weak solutions (e.g. 0·05%), but a 100% block of the γ-δ-fibres was never achieved without some reduction in the α-wave. Experiments conducted simultaneously on ventral and dorsal roots indicate that there is no essential difference in the rate of blocking of motor and sensory fibres of comparable conduction velocity.

With weak solutions of procaine it was possible to abolish completely the C-fibre potential before any depression of the α-wave occurred, although by this time the γ-δ-elevation was reduced. α-Fibres always recovered before γ- and δ-fibres but the C-elevation was more variable in its time for recovery.

Since the ratio of surface area to volume is greatest for small fibres the concentration of local anaesthetic within them will rise more rapidly towards the external concentration—if the process is one of diffusion. If it is supposed that a critical concentration is required somewhere along the diffusion path in order to procure block then this value would be reached more rapidly in the smaller fibres; this is what is found experimentally.

The effect of the activity of the γ-motoneurones on the relation between tension and extension in the stretch reflex.

By P. B. C. Matthews. *University Laboratory of Physiology, Oxford*

When the γ-efferent nerve fibres to the soleus muscle of a decerebrate cat are selectively paralysed then the stretch reflex of soleus, evoked by a standard extension, becomes smaller or is abolished (Matthews, 1957; Matthews & Rushworth, 1957). The stretch reflex evoked by a standard extension represents, however, only one point on a curve relating the stretch reflex tension to the length of soleus. In the present experiments the role of the γ-motoneurones in the stretch reflex has been studied by determining the effect on this tension–length relationship of selectively paralysing the γ-efferents by applying procaine to the nerve to soleus.

Cats decerebrated either by mid-brain section or by anaemia were used. The relation between the tension in soleus and its length was determined in a single operation by extending soleus (by 10–15 mm) at a slow constant rate (0·5–2 mm/sec) and simultaneously recording both its length and tension. The 'active tension' developed at successive increments of length was determined from the total tension, by subtracting the 'passive tension' (obtained later when reflex absent). The 'active tension' was then plotted against length. The tension–length curve determined by this means must depend upon both 'static' and 'dynamic' properties of the stretch reflex arc. At the slow rates of stretching used the 'static' properties appear to have been predominant, as varying the rate of stretching had little effect on the curve.

The active tension usually increased progressively with length, though occasionally it fell when the extension was large. At small extensions the tension–length curve tended to be concave upwards, but at larger extensions the curve usually became linear over a range of several mm. Granit & Stark (1957, personal communication) have made similar observations on the stretch reflex evoked by static extensions.

While the γ-efferents were being paralysed the tension–length curve altered progressively so that increasing extensions were required to excite any particular reflex tension. But the curve usually remained linear at large extensions and the slope of this linear portion showed little or no change. The whole curve, however, shifted to the right and for any particular extension the decrease of the stretch reflex was due to the shift of the curve, rather than to alteration of its slope. Sometimes active tension persisted at the shortest lengths used; but sometimes the active tension was abolished at short lengths though still appearing at greater lengths, that is, the stretch reflex had developed a threshold (of extension). Thus in decerebrate cats the activity of the γ-motoneurones affects the 'threshold' rather than the 'stiffness' of the stretch reflex.

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The ventilatory response to a rise in body temperature in relation to other physiological variables. By J. E. Cotes and F. Meade. *Pneumoconiosis Research Unit of the Medical Research Council, Llandough Hospital, Penarth, Glam.*

Indices are presented for eleven male volunteers 18–19 years old, calculated on the assumption (cf. Gray, 1950; Bannister, Cunningham & Douglas, 1954; Cotes, 1955) that, for subjects performing steady-state exercise and breathing oxygen, ventilation can be expressed in the form $\dot{V}_E = a\mathrm{pCO_2} + b\,\textsc{b.t.} + c$ over the physiological range of these variables ($\textsc{b.t.}$ = body temperature, °C).

Estimates were made of body-surface area (Sendroy & Cecchini, 1954), pulmonary diffusing capacity (Bates, Boucot & Dormer, 1955) and steady-state oxygen consumption, rectal temperature and ventilation at two levels of end tidal $\mathrm{pCO_2}$ whilst breathing mixtures of $\mathrm{CO_2}$ in oxygen. Studies at rest and whilst walking at 3·5 m.p.h. (5·6km/hr) were performed on two occasions as part of an investigation into the effects of training (Cotes, 1956). These results have been combined since there are no significant differences between the two sets of data.

The ventilation increases per unit increase in $\mathrm{pCO_2}$ at the resting and exercise body temperatures do not differ significantly. The recorded increases per unit increase in $\mathrm{pCO_2}$ and in body temperature (Table 1) are derived from the pooled data; these indices differ between subjects. Other data are presented as average values with their standard errors.

On average, the increase in ventilation over its initial value ($\Delta\dot{V}_E$) can be described by the relationship:

$$\Delta\dot{V}_E = (1{\cdot}5 \pm 0{\cdot}38)\,\Delta\mathrm{pCO_2} + (14{\cdot}7 \pm 1{\cdot}31)\,\Delta\textsc{b.t.}\ \text{l./min}\ \textsc{b.t.p.s.}$$

This relationship accords with other estimates.

TABLE 1

Subject	Ventilation increase per mm. increase in pCO_2	Ventilation increase per ° C increase in body temp.	Body temp. increase per cal. increase in energy expenditure ±0·0276	Ventilation equivalent l./100 ml. O_2		D_{CO} ml./min/ mmHg ±3·48	Body surface area (m²)
				Rest ±0·241	Exercise ±0·0960		
1	1·373	26·786	0·119	3·02	2·386	39·3	1·84
2	1·219	28·433	0·143	2·70	2·108	46·4	1·79
3	1·464	11·642	0·283	3·07	2·427	28·0	1·60
4	2·389	15·146	0·254	3·32	2·322	25·3	1·65
5	1·690	—	0·089	3·42	2·298	31·0	1·77
6	2·106	12·172	0·230	2·78	2·380	29·5	1·61
7	1·471	11·938	0·245	2·39	2·179	30·9	1·54
8	1·578	8·496	0·267	2·71	2·425	29·7	1·62
9	1·061	25·736	0·138	2·53	2·179	36·3	1·64
10	1·854	21·759	0·161	3·25	2·249	36·8	1·89
11	1·212	16·526	0·245	2·82	2·120	28·8	1·64

The increase in body temperature per calory increase in energy expenditure is negatively correlated ($r = -0{\cdot}74$) with body-surface area (cf. Robinson, 1942).

The increase in ventilation per unit increase in body temperature is positively correlated ($r = 0{\cdot}73$) with body-surface area. The data are not sufficient to define the form of these relationships.

We are indebted to the Army Personnel Research Committee for their co-operation and to Mr P. D. Oldham for statistical advice.

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The antagonism, by thyroid hormone, of the early onset of muscular fatigue in adrenalectomized salt-maintained animals.

By S. N. GANGU and MARY F. LOCKETT. *Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.* 3

The rate of onset of both neuromuscular and of muscular fatigue has been measured, under urethane anaesthesia, in salt-maintained adrenalectomized male white mice, 20–30 g, on the seventh post-operative day. The methods used were a modification of Ingle's work test (1944), and the results obtained with adrenalectomized mice confirm and extend Ingle's findings in salt-maintained adrenalectomized rats. Rectangular stimuli, 0·35 msec in duration, were delivered each second from a 10 V source either to a sciatic nerve peripheral to a ligature, or directly to a gastrocnemius muscle. The contractions evoked were recorded on a smoked drum by a lever attached by a thread to the Achilles tendon. Pre-treatment for 6 days with either 3–6 mg dried thyroid orally/day, or with 0·25 μg L-thyroxine or 0·25 μg L-triiodothyronine by injection/day, caused prolongation by 50 % of the period for which conduction continued at the myoneural junction and of the time for which muscles were able to respond to direct stimulation by lifting a 5 g weight through ⅓ mm. Similar treatments with either larger or smaller doses of thyroid hormone were decreasingly effective. Thus the dose–effect curve for these two actions of thyroid hormone was approximately parabolic.

Thirty minutes' exposure of isolated rat phrenic nerve diaphragm preparations to 0·05 μg L-triiodothyronine/100 ml. Tyrode soln. did not change the maximal or submaximal responses to electrical stimulation delivered either through the nerve or directly to the muscle. This concentration of hormone did, however, in less than 15 min, significantly antagonize block of the myoneural junction by excess of potassium ions, but scarcely effected potassium block of the effects of direct muscle stimulation. Visscher (1950) has previously reported the rapid action of thyroid hormone on phrenic nerve diaphragm preparations. Incubation of such preparations from thyroidectomized rats with thyroxine 3×10^{-7} for 1 hr restored their sensitivity to adrenaline to normal in the presence or absence of potassium in the Tyrode solution used.

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The effects of adrenaline, noradrenaline, and isoprenaline, on urinary excretion in unanaesthetized rats.

By Mary F. Lockett and Regina M. Mrozowska. *Department of Physiology and Pharmacology, Chelsea College of Science and Technology, London, S.W.* 3

Subcutaneous injections of 2·5–25 μg adrenaline/100 g/rat delayed the excretion of water loads (5 % of body weight). This antidiuretic action of adrenaline occurred without change in either mean arterial pressure or in inulin clearance, and was accompanied by decrease in the concentrations of both sodium and potassium ions in the urine. Neither the removal of the posterior lobe of the pituitary body, nor adrenalectomy, nor sensory denervation of the injection area abolished the antidiuretic action of adrenaline. This antidiuresis was, however, reduced by substitution of a 4 % alcohol for a water load. Larger doses of adrenaline, 60–120 μg/100 g body weight, had diuretic action, as had noradrenaline in doses of 2·5–5·0 μg/100 g. Isoprenaline was antidiuretic throughout the dose range of 0·1–5·0 μg/100 g weight.

In normal rats 0·25 μg isoprenaline/100 g body weight was more effective as an antidiuretic, but was less effective in causing retention of sodium and potassium ion, than was 5 μg adrenaline/100 g. Two to three weeks after post-ganglionic denervation of the kidneys, the sodium retention caused by 5 μg/100 g adrenaline was either absent or was much reduced, but the effect of this dose on potassium retention was unchanged. By contrast, this denervation of the kidneys did not diminish either the antidiuretic action, or the sodium and potassium retention caused by 0·25 μg isoprenaline/100 g weight. Inulin clearances were not significantly altered by the denervation.

The cell membrane concept.

By J. D. ROBERTSON. *Department of Anatomy, University College London*

Several layers of varying nature measuring from several hundred Ångström units down to ~30 Å have been seen by electron microscopists at the surfaces of cells and referred to as the 'cell membrane'. It is important to decide whether or not any of these layers or combinations of layers is common to all cells and should be designated rigorously as the cell membrane. This might be expected (Davson & Danielli, 1943) to be a structure 50–100 Å across, consisting of definite molecular layers. A unit such as this measuring ~75 Å across, consisting of two parallel dense lines ~25 Å wide separated by a light zone ~25 Å wide, will be shown to be characteristic of the surfaces of a wide variety of cell types. These include nerve, skin, liver, pancreas, kidney and endothelial, blood and muscle cells of vertebrates. The unit is also seen in cells of animals of other phyla. It is constantly present in the different double membranes of vertebrate cell organelles including mitochondria, endoplasmic reticulum and nuclear membranes. In the special case of Schwann cells this unit is shown to constitute half of each myelin lamella and hence probably at least contains one bimolecular leaflet of mixed lipids (Robertson, 1957*a*).

It will be shown that these unit membranes assume definite relationships to one another in the general case of double membranes, and the specific case of neuromuscular synapses. In certain double membranes it is possible to alter experimentally (Robertson, 1957*b*) these appositional relationships by means of solutions of different tonicities, so that the ~100–150 Å gap between the unit membranes may be closed or widened. This finding, besides pointing to the stability of the ~75 Å units, suggests that a highly hydrated gel may be present in the gap of some double membranes.

On the basis of these observations it appears that an entity of definite morphology is present at the surfaces of a sufficiently wide variety of cell types to allow this structure to be defined tentatively as the cell membrane. Additional closely associated but variable structures may be considered as specializations added to this basic structural unit.

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The production of regenerative responses in crayfish muscle fibres by the action of calcium, strontium and barium.

By P. Fatt and B. L. Ginsborg*. *Biophysics Department, University College London*

Investigations on muscle fibres of marine crustaceans have revealed that action potentials can be produced in Na-free solution after the muscle has been soaked in a solution containing certain quaternary ammonium ions (Fatt & Katz, 1953). Since the only cations present in the final solution were Ca, K and Mg, the possibility arose that an inward movement of Ca or Mg across the fibre surface might provide the flow of electric charge necessary to displace the potential across the membrane capacity and balance the current carried by K and Cl.

Fig. 1. A. Response of crayfish muscle fibre in 20 mM CaCl$_2$–210 mM NaCl to an outward current pulse. Intracellular electrodes for applying current and recording potential close together. B. Action potential of muscle fibre in 160 mM SrCl$_2$. Stimulating current pulse applied several mm away from position of recording. C. Action potential of muscle fibre in 160 mM BaCl$_2$. Complete record included in 3 sweeps. In A and B upper horizontal line is potential recorded outside the fibre. Duration of current pulse indicated by horizontal bar. Same voltage scale for all records. Note sweep speed of C 1/10th that of A and B.

The response of a crayfish muscle fibre in 20 mM CaCl$_2$ + 210 mM NaCl is illustrated in Fig. 1A. It consists in a non-propagated damped oscillation superimposed on a large passive (i.e. electrotonic) depolarization. Removal of Na from the solution (replacing NaCl by glycine) or addition of Mg up to 50 mM (MgCl$_2$ partly replacing NaCl) had no effect on the response. In contrast, an increase in the concentration of Ca up to 50 mM increased the amplitude of the oscillation. The response could often be reduced by removing Ca from the solution; it was always abolished by the addition of citrate, which indicated that washing the muscle with Ca-free solution did not completely remove Ca from the immediate environment of the fibre.

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When Ca was replaced by Sr, large propagated action potentials were elicited. The amplitude and duration of the action potential increased when the concentration of Sr in the bathing solution was raised. Fig. 1 B shows the action potential obtained from a fibre in a solution consisting simply of 160 mM $SrCl_2$. Muscles soaked for 20 hr in this solution yielded similar action potentials. As illustrated in Fig. 1 C, much more prolonged action potentials were elicited in 160 mM $BaCl_2$. The action of Sr and Ba was reversible.

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